

Abstracts

POSTER PRESENTATIONS



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Jennifer Lippincott-Schwartz President / **Wallace Marshall** Program Chair

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POSTER PRESENTATIONS- Sunday, December 7

Imaging Technologies, Single Molecule Imaging, and Super-Resolution 1

P1

Board Number: B100

Development of three color variants of super-brilliant luciferase for multi-color, real time imaging of gene expression and dynamics of organelles and the cytoskeleton without external illumination.

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Since bioluminescence does not require excitation light, it is free from auto-fluorescence. Therefore, it has been used for quantitative analysis of gene expression and in vivo imaging. Furthermore, it is free from potential phototoxicity and is compatible with optogenetic tools. However application of bioluminescent imaging has been limited mainly by two drawbacks. Firstly, the light output from the bioluminescent protein or luciferase was much darker than fluorescent proteins (FPs), making it difficult to study fine structures such as cytoskeletal dynamics with luminescent imaging. Secondly, color variants of luciferase have been limited compared with FPs, precluding multicolor imaging.

In our recent study, we addressed the first limitation by developing a super brilliant yellow luminescent protein, Nano-lantern (Saito et al., *Nat. Commun.* 2012). In this study, we have overcome the second barrier. We report the development of cyan and orange variants of Nano-lantern, both of which are even brighter than the original yellow Nano-lantern by 1.5-2.3 times, and bright enough for observation with the naked eye or with a smartphone camera. Fusions of these multicolor Nano-lanterns with a variety of subcellular localization tags showed correct localization in both luminescent and fluorescent imaging, demonstrating their utility as imaging probes. In addition, expansion of the color palette of Nano-lanterns enabled not only multicolor luminescent imaging of subcellular structures, but also expression analysis of multiple genes at single cell level in embryonic stem cells, which are known to be very sensitive to phototoxicity. Furthermore, by combining split luciferase complementation with Ca²⁺-sensing peptide (CaM-M13), we demonstrated simultaneous measurement of Ca²⁺ dynamics in the nucleus and mitochondria.

Our multicolor Nano-lanterns are expected to be excellent imaging tools for gene-expression analysis, in vivo imaging, stem cell study and combinatorial analysis with optogenetics. Considering that the expanded color palette of FPs has driven wider application of fluorescent live imaging, we believe that

our multicolor Nano-lanterns should change the world of luminescent imaging from dim and monochrome to extremely bright and colorful.

P2

Board Number: B101

Development of novel in situ and in vivo RNA detection methods based on FIT probes.

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We have been developing new tools based on fluorogenic forced intercalation (FIT) probes for RNA detection quantification and interference in biological samples. Upon duplex formation with target nucleic acids, the base surrogates TO dye increases its quantum yield and brightness substantially (>10 fold). We turned FIT probes brighter by including a second, brighter, slightly red shifted variant dye, JO, which is hardly responsive. In this setup TO acts as a light harvester that feeds JO - we showed that a single FIT probe is sufficient to allow detection of oskar mRNA in a rapid, wash free FISH setup using conventional wide-field microscopy, making it an ideal tool for RNA localization screens.

Locked nucleic acids (LNA) immediately adjacent to the TO base surrogate also enhance brightness – without significantly affecting responsiveness – by shortening the distance between stacked nucleotides in duplexes. This unique behavior of FIT probes allows synthesis of nuclease resistant oligos that are bright and contrasted enough for use in live imaging: as few as two LNA-containing FIT probes targeting different segments of oskar mRNA are sufficient to monitor oskar RNP motility in living oocytes. Motility data obtained by using 3-5 different probes in wild-type oocytes recapitulate what has been reported with the oskMS2-GFP system, indicating that this is a viable, non-transgenic alternative for studying mRNP biogenesis in vivo.

While normally the aim is to avoid interference with biological processes, nuclease-resistant oligos can also be targeted against RNA secondary structures and/or protein binding interfaces of interest. FIT probes, in addition, report successful hybridization, allowing the testing of the functional role of RNA segments. We find that a FIT probe targeting the 3' of the proximal stem of the spliced oskar localization element (SOLE) that is essential for efficient kinesin-dependent transport and oskar localization, causes motility defects nearly identical to those observed when mutating the SOLE sequence in transgenic oskar mRNAs.

Finally, we demonstrated that TO fluorescence reaches its maximum at stoichiometric probe-to-target ratio, and equipping FIT probes with a Cy7 presence reporter we could quantify oskar mRNA concentrations in situ. Although this qFIT method lacks single molecule sensitivity at the moment, it can measure high RNA concentration - even hundreds of RNA molecules within a confocal unit - providing a complementary approach to single molecule FISH studies.

P3**Board Number: B102****Co-opting fluorescent proteins for cell-specific gene manipulation.**C. Tang¹, T. Szikra², Y. Kozorovitskiy³, M. Teixeira², S. Lapan¹, B. Sabatini³, B. Roska², C. Cepko¹;¹Genetics and Ophthalmology, Harvard Medical School/HHMI, Boston, MA, ²Neural Circuit Laboratories, Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland, ³Neurobiology, Harvard Medical School/HHMI, Boston, MA

Studies of the cell biology of multicellular organisms would be greatly facilitated by the ability to manipulate gene expression in any desired cell type. However, it remains a challenge to achieve cell-specific gene manipulation in many model systems, especially the mouse nervous system. Fluorescent proteins are commonly incorporated into transgenic organisms for labeling specific cell populations, but the unmodified forms cannot control biological activities. Using GFP-binding proteins derived from Camelid antibodies, we co-opted GFP as a scaffold for inducing formation of biologically active complexes, developing a library of hybrid transcription factors that control gene expression only in the presence of GFP or its derivatives. The modular design allows for variation in key properties such as DNA specificity, transcriptional potency, and drug dependency. Production of GFP controlled cell-specific gene expression and facilitated functional perturbations in the mouse retina. Further, retrofitting existing transgenic GFP mouse and zebrafish lines for GFP-dependent transcription enabled applications such as optogenetic probing of neural circuits. Current efforts to expand GFP-regulated activities, such as for GFP to directly control Cre recombination, will be discussed. This work establishes GFP as a multifunctional scaffold and opens the door to selective manipulation of diverse GFP-labeled cells across transgenic lines. This approach may also be extended to exploit other intracellular products as cell-specific scaffolds in multicellular organisms.

P4**Board Number: B103****“Decorating” cells with genetically encoded fluorescent proteins – what color suits you best?**J. Monbo¹, L. Tu², A. Joseph², O. Ospovat¹, A. Myers¹, D. Grunwald²;¹RNA Therapeutics Institute, University of Massachusetts Medical School, Worcester, MA, ²RNA Therapeutics Institute, University of Massachusetts Medical School, Worcester, MA

A key aspect of simultaneous multicolor single molecule real-time imaging in the living cell is choosing the appropriate combinations of fluorescent proteins. While ample information is available on molecular brightness, photo-stability, pH sensitivity and monomeric nature of most fluorescent proteins, interpolating the published data into performance in mammalian live cell experiments has been challenging. This leads to situations where the effort of testing new fluorescent proteins, combined with re-cloning of genetic constructs favor the use of suboptimal fluorescent protein combinations. The price paid is that less signal is collected using higher excitation power shortening the observation time of the

fluorescent protein. Additionally, higher excitation can also mean photo-damage to the cell thereby inducing a 'stress' reaction, potentially compromising results. Using a list of parameters, we tested 38 different fluorescent proteins for their suitability in live cell single molecule imaging. We refer to this set as a 'toolbox', and we have systematically evaluated all the fluorescent proteins for labeling of mobile (diffusing fluorescent protein & diffusive fusion protein) and immobile molecules (nuclear pore marker). We pay particular attention to the amount of fluorescence signal under low excitation power imaging conditions, sub-cellular (mis)-localization, cross excitation and cross emission at non-maximal photon output. This 'toolbox' will be of great use to the microscopy community.

P5

Board Number: B104

A Live Cell Antifade Solution for GFP Imaging.

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Time-lapse, fluorescent imaging is a powerful tool in cell biology research. Photobleaching is one of the largest barriers for longer term imaging of fluorescent proteins and dyes. This phenomenon is caused by exposure to the intense light required to excite them. The loss of intensity of the fluorescent signal can leave researchers with incomplete or unclear data. Measures taken to minimize light exposure should be designed into each time-lapse experiment (Brown et al., 2009 and Waters, 2013). Factors to consider when designing these time-lapse, fluorescent experiments are light exposure time, the intensity of the light, and frequency of sampling (Brown et al., 2009) which can decrease the effects of photobleaching in an experiment. In addition, novel, oxygen-scavenging methods can also be used to reduce the amount of photobleaching observed in these types of experiments. Here is a study of a set of compounds analyzing their effectiveness at reducing photobleaching of fluorescent proteins and fluorophores. Results from a model system using GFP, RFP, and fluorescent dyes have led to a solution which reduces photobleaching in GFP, RFP, and several other dyes.

P6

Board Number: B105

A palette of near-infrared fluorescent proteins.

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The genetically encoded near-infrared fluorescent probes are preferable for non-invasive in vivo imaging. In the near-infrared spectral region (650–900 nm) mammalian tissues are relatively transparent to light because the combined absorption by hemoglobin and water is minimal. Previously, we have

developed five spectrally distinct fluorescent proteins, such as iRFP670, iRFP682, iRFP702, iRFP713 (aka iRFP) and iRFP720, from bacterial phytochromes (1, 2). As a chromophore, iRFPs use a heme derivative, called biliverdin, abundant in mammalian cells. All iRFPs incorporate endogenous biliverdin efficiently and autocatalytically, do not require its exogenous supply and, therefore, can be used as easily as GFP-like proteins. iRFPs are dimers and can mainly serve for labeling of organelles and whole cells. iRFPs have enabled multicolor imaging of deep tissues in living animals.

Here, we report the development of monomeric iRFPs (miRFPs) suitable for protein tagging, which also do not require external biliverdin to fluoresce. To engineer miRFPs we applied a combination of the rational and random mutagenesis, followed by a high-throughput screening. As a result, we have obtained three spectrally different monomeric near-infrared proteins, named miRFP670, miRFP703 and miRFP708. miRFPs are characterized by a high effective brightness in mammalian cells, a high pH stability and a high photostability. We demonstrated that miRFPs perform well as fusion tags for cellular proteins. The set of miRFPs should enable imaging of several tagged proteins in living mammals, and thus will be useful in cell and developmental biology and biomedicine. The developed rational design should allow efficient engineering of future miRFPs with desirable spectral and biochemical properties.

(1) Filonov G.S. et al., Bright and stable near infra-red fluorescent protein for in vivo imaging. *Nature Biotechnology* 2011, 29: 757-761. (2) Shcherbakova D.M. and Verkhusha V.V. Near-infrared fluorescent proteins for multicolor in vivo imaging. *Nature Methods* 2013, 10: 751-754.

P7

Board Number: B106

Two-color imaging using spectral variants of iRFP670 and iRFP682 near-infrared fluorescent proteins.

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The new class of fluorescent proteins (FPs) engineered from bacterial phytochromes (BphPs) [1] attracts much attention for in vivo imaging due to their near-infrared (NIR) fluorescence spectra. These FPs utilize widely available in mammalian cells biliverdin (BV), a product of heme degradation, as a chromophore and, therefore, are as easy to use as common GFP-like proteins. We recently reported five NIR FPs, called iRFPs, with different fluorescence and biochemical properties [2]. Interestingly, two of them, iRFP670 and iRFP682, exhibited the twice higher molecular brightness, as well as the blue-shifted absorbance (643 nm and 663 nm) and fluorescence (670 nm and 682 nm) compared to other iRFPs. Here we characterized the unusual properties of these NIR FPs in detail. Our biochemical and biophysical analysis showed that iRFP670 and iRFP682 incorporate the BV chromophore in two distinct confirmations. A single amino acid mutation resulted in a depletion of one BV confirmations in the protein binding pocket and, consequently, in 30 nm red-shifts of both absorbance and fluorescence. The

point mutation also caused a slight decrease in the molecular brightness and an increase in the pH stability of the obtained red-shifted variants, which we named iRFP670-red and iRFP682-red. The effective brightness of the iRFP670-red and iRFP682-red in live mammalian cells was comparable to that of the parental proteins, suggesting that the high efficiency and high specificity of the incorporation of endogenous BV chromophore was not affected. Spectrally resolvable fluorescence of the iRFP670 and iRFP670-red pair, as well as of the iRFP682 and iRFP682-red pair, allowed easy separation of two cellular populations using FACS cytometry and straightforward two-color fluorescence microscopy of live cells, thus making them the probes of choice for cell labeling in the NIR region. Our studies are also beneficial for a rational molecular design of future enhanced NIR FPs from a variety of natural BphPs.

[1] Piatkevich K.D., Subach F.V. and Verkhusha V.V. Engineering of bacterial phytochromes for near-infrared imaging, sensing, and light-control in mammals. *Chem. Soc. Reviews* 2013, 42: 3441-3452. [2] Shcherbakova D.M. and Verkhusha V.V. Near-infrared fluorescent proteins for multicolor in vivo imaging. *Nature Methods* 2013, 10: 751-754.

P8

Board Number: B107

Minimal tags for rapid dual-color live cell labeling and super-resolution microscopy.

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The choice of appropriate labeling technique is crucial for optimal super-resolution microscopy (SRM). Labeling density, probe size and photophysical properties are particularly important and to great extent determine the power of SRM. In addition, the probe should be attached as close as possible to the target protein as large linkers can induce artifacts and lower resolution. Many organic fluorophores offer small size and good photophysical characteristics, especially when compared to widely used GFP variants, but their direct linking to the target proteins remains challenging. We present a method that permits direct coupling- with virtually no linker- of SRM compatible dyes site-specifically to the target proteins by combining Amber codon suppression and click-chemistry technologies. To achieve this, host cells are modified to incorporate unnatural amino acids (UAAs) in response to a unique codon during translation. These UAAs carry chemical groups (strained alkynes and alkenes) that can be directly attached to tetrazine functionalized dyes in a special type of a fully biocompatible click-chemistry reaction named the inverse-electron-demand Diels-Alder cycloaddition (SPIEDAC). By testing different tetrazine derivatives of organic dyes, we tuned the SPIEDAC reaction in an orthogonal descendant for rapid dual-color protein labeling. These two reactions allowed labeling of distinct insulin receptor populations and Influenza VLPs (virus-like particles) with two different dyes in living mammalian cells, as shown by SRM. While we demonstrate the applicability of our tools for SRM, generic SPIEDAC ligation mechanism is transferrable to other techniques and could e.g. be used for making PET/MRI imaging tracers.

P9**Board Number: B108****Multicolor Fluorescence Photoactivation Localization Microscopy by Spectral Measurements.**M.J. Mlodzianoski¹, S. Gunewardene¹, N.M. Curthoys¹, S. Carter¹, S.T. Hess¹;¹Department of Physics and Astronomy, University of Maine, Orono, ME

The localization-based super resolution microscopy techniques Fluorescence Photoactivation Localization Microscopy (FPALM) (Hess et. al. 2006), Photoactivatable Localization Microscopy (PALM) (Betzig et. al. 2006), and Stochastic Optical Reconstruction Microscopy (STORM) (Rust et. al. 2006) circumvent the diffraction limit by measuring subsets of single molecules over a period of time. Spatially separated single molecules can then be localized with an uncertainty much smaller than the diffraction-limited resolution of the microscope. Reconstruction of images using localized single molecule positions can achieve a final image resolution on the order of tens of nanometers, a roughly ten-fold improvement over the diffraction limited resolution of ~250 nm.

Imaging single molecules records information that is lost in ensemble fluorescence microscopy techniques. This single molecule information has enabled additional imaging modalities and has led to three-dimensional super-resolution imaging (Huang et. al. 2008, Juetten et. al. 2008), dipole orientation (Gould et. al. 2008), and imaging of multiple fluorescent species (Huang et. al. 2007, Shroff et. al. 2008, Gunewardene et. al. 2011). Multicolor techniques are especially important in biological applications because they allow for the direct comparison of the spatial distribution of two or more differently labeled structures.

Here, we describe a novel multicolor imaging method that measures the emission spectrum of each single molecule directly. Fluorescence is split into two channels with a standard 50:50 beamsplitter; one channel measures the spatial distribution while the second channel is sent through a dispersive optic. Within the spectral channel, the molecular image is shifted and broadened according to its emission spectrum. Quantification of the emission spectrum allows for identification of the fluorescent species. Previously, ratiometric simultaneous multicolor images were limited to four or fewer different species if the collected number of photons was large and background noise low. Depending on the background noise, number of photons collected, and the width of the spectral window, our spectral method can distinguish a larger number of species simultaneously. Additionally, a large set of novel super-resolution imaging methodologies can be envisioned, based on changes in the emission spectra of fluorescent molecules as a function of pH, temperature, hydrophobicity, time, or any method which induces fluorescence emission spectra changes in the sample.

P10**Board Number: B109****Nanometer Resolution with the Time-resolved Confocal Microscope MicroTime 200.**

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Superresolution microscopy is about to evolve into a standard tool for biological research. Overcoming the diffraction limit for fluorescence imaging has been shown to be crucial for addressing various relevant biological questions. However, the resolution is practically limited to about 20 nm for STED, STORM or PALM. Optical resolution below 20 nm can be easily achieved by combining confocal detection with AFM tip induced local fluorescence modulation. With FRET, distances even below 10 nm are readily accessible.

As an open confocal microscopy platform, the MicroTime 200 is the ideal tool to combine sub diffraction spatial resolution with ultrasensitive time-resolved detection. Picosecond pulsed lasers and confocal detection together with time-correlated single photon counting (TCSPC) allow this system to add the full benefit of fluorescence lifetime based experiments to address highly specific biological questions.

We present combined Confocal+AFM measurements on the single molecule level with DNA Origami structures proving a resolution of even below 10 nm. We show fluorescence lifetime based FRET imaging (FLIM FRET) to be a superior approach in the case of concomitant FRET subpopulations enabling the extraction of accurate FRET efficiencies and donor acceptor distances in the nanometer range.

For multi species cell labeling we developed a pattern matching based approach which can take spectral as well as fluorescence decay information into account to improve the label recognition and separation especially in complex cellular environments. Finally the time-resolved single photon analysis is also applied to diffusion based FCS measurements in live cells to identify parasitic autofluorescence contributions on the nanosecond timescale which otherwise hamper the extraction of accurate local concentrations.

Keywords: confocal microscopy, TCSPC, FLIM, FCS, FRET, AFM, single molecule, pattern matching

P11**Board Number: B110****Video-rate super resolution microscopy in living cells.**F. Huang¹, C.E. Laplante², Y. Lin³, T.D. Pollard^{1,2,4}, J. Bewersdorf¹;¹Department of Cell Biology, Yale University School of Medicine, New Haven, CT, ²Department of Molecular, Cellular and Developmental Biology, Yale University School of Medicine, New Haven, CT,³Department of Biomedical Engineering, Yale University School of Medicine, New Haven, CT,⁴Department of Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, CT

Super resolution microscopy based on single molecule localization relies on precise and accurate localization of large numbers of single molecules. However, the necessity of accumulating large numbers of localization estimates limits the time resolution typically to seconds to minutes^{1,2}.

Two major limitations are the acquisition speed and the photon budget. An EMCCD camera is usually used in such experiments. Replacing it with a recently introduced sCMOS camera results in leaps in both acquisition speed and effective quantum efficiency. However, the intrinsic pixel-dependent Gaussian noise of the sCMOS cameras introduces localization artifacts and greatly reduces the reliability of the results.

Here, we present a set of specially designed statistics-based algorithms that characterize an sCMOS camera for the first time and allow for unbiased and precise localization analysis. Using this method we demonstrate Cramer-Rao lower bound-limited single molecule localization with an sCMOS camera. Combining the novel algorithm with a recently developed multi-emitter fitting algorithm³ and optimized imaging condition, we show that this technique shortens the typical acquisition time for fixed samples by up to two orders of magnitude without compromising the field of view. Furthermore, we demonstrate localization-based super-resolution microscopy in live cells by monitoring dynamics of protein clusters, vesicles and organelles at a temporal resolution from 2 to 30 frames per second⁴.

These methods allowed us to investigate cytokinetic apparatus in live fission yeast at 20-30 nm resolution. In general, the significantly improved temporal resolution allows super resolution imaging of a large range of dynamic events in living cells.

Patterson, G., Davidson, M., Manley, S. & Lippincott-Schwartz, J. Superresolution imaging using single-molecule localization. *Annu. Rev. Phys. Chem.* **61**, 345–67 (2010).

Gould, T. J., Hess, S. T. & Bewersdorf, J. Optical nanoscopy: from acquisition to analysis. *Annu. Rev. Biomed. Eng.* **14**, 231–54 (2012).

Huang, F., Schwartz, S. L., Byars, J. M. & Lidke, K. A. Simultaneous multiple-emitter fitting for single molecule super-resolution imaging. *Biomed. Opt. Express* **2**, 1377–93 (2011).

Huang, F. et al. Video-rate nanoscopy using sCMOS camera-specific single-molecule localization algorithms. *Nat. Methods* **10**, 653–8 (2013).

P12

Board Number: B111

Ultrafast super-resolution fluorescence live imaging with spinning disk confocal microscope optics.

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Most current super-resolution (SR) microscope techniques surpass the diffraction limit at the expense of temporal resolution, compromising their applications to live cell imaging. Here, we report a new SR method based on the spinning-disk confocal microscope that achieves a spatial resolution of about 120 nm, double that of conventional microscopy. Theoretically, our method is equivalent to the structured illumination microscopy (SIM), but it is 10 times faster. Conventional SIM reconstitute a SR image from 9-15 raw images. The SR signals are modulated as Moire patterns in the raw images, and are extracted by the digital processing. Our method recovers the SR signals by the optical demodulation through the stripe pattern of the disk, and only a single averaged image through the rotating disk is required for a single SR image. Thus, the frame rate of our method is only determined by the speed of the disk and the camera. Based on this theory, we have modified a commercial spinning disk confocal microscope. The improved resolution around 120 nm was confirmed with biological samples such as nuclear pores or microtubules. The rapid dynamics of EB1 at the growing ends of the microtubules were observed with 10 ms image acquisition time. Since our method requires only little optical modifications, it will enable an easy upgrade from existing spinning disk confocal to a SR microscope for live cell imaging.

P13

Board Number: B112

Device for microwave-enhanced processing of cryo-substitution for light and electron microscopy.

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Cryo-substitution of frozen hydrated samples is the premier method for preserving native structure of specimens sectioned for light and electron microscopy. Also called freeze substitution, sample processing involves replacing ice with fixatives in organic solvents at or below -80 degrees C, followed by gradual warming to permissive temperatures for chemical cross-linking, typically over a 2-5 day period. In order to facilitate substitution and fixation, we developed and tested a cryo-thermal device for regulating sample temperature within standard laboratory microwave processors. The system consists of a microwave processor modified to accommodate a cryo-thermal sample platform and an external

programmable controller. Time, temperature, transition triggers, and ramping rates are user-defined between ± 100 degrees C, and recorded in spreadsheet format during processing. Using the system, we processed high-pressure frozen *Bacillus subtilis*, *Saccharomyces cerevisiae*, *Borrelia burgdorferi*, mixed pond water biota, Plasmodium-infected erythrocytes, and Chlamydia-infected HeLa cells in under 90 min. Embedding in acrylic resins in the platform at -20 or -40 degrees C added about another hour. Cooling and heating rates exceeded 20 and 10 deg/min, respectively. Recorded sample temperature plateaus varied less than 0.5 degree. Structure, contrast, tomography, and immune labeling were equivalent or superior to that achieved by traditional cryo-substitution or standard ambient-temperature fixation. Membrane contrast was reversible by adjusting the temperature at which samples were transferred from fixative mixtures to solvent only. Equivalent fixative mixtures produced negative contrast of mitochondrial cristae when transferred out of fixative at -20 degrees, and positive contrast when transferred at 20 degrees C. The system also enabled sequential use of three separate fixatives, with washing steps, in approximately 3 hours. These results indicate consistent, flexible, efficient, and effective methods for preparing and preserving diverse cryo-samples in near native state for light and electron microscopy.

P14

Board Number: B113

Title: Permeabilization Activated Reduction in Fluorescence (PARF): a novel method to measure kinetics of protein interactions with intracellular structures.

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Understanding kinetic information is fundamental in understanding biological function. Advancement in imaging technologies have allowed for the development of assays for kinetic analysis. Fluorescence recovery after photobleaching (FRAP) is one such method that can be used to determine the diffusion rate of fluorescently labeled proteins in cellular environments and the kinetics describing interactions with intracellular structures. Through examination of the recovery kinetics of a photobleached region of interest, a researcher can determine the apparent exchange rate of the bleached fluor and half-life for recovery under equilibrium conditions. Like many of the other experimental approaches using biophotonic analysis to gain kinetic insight, FRAP requires a significant investment in specific equipment. We have developed a technique to measure kinetic information that does not require such a specialized imaging setup. Permeabilization Activated Reduction in Fluorescence (PARF) is an imaging-based kinetic analysis that allows the user to determine an apparent off-rate for a fluorescently-tagged protein of interest. To create conditions where the off-rate is observable, cells expressing a fluorescently-tagged protein are permeabilized with 25 μ M digitonin. The plasma membrane becomes permeable to molecules up to 200kD in size, allowing for the unbound pool of labeled protein in the cytosol to diffuse into the media. As the media volume is much larger than the cytosolic volume, the concentration of the unbound pool decreases drastically, shifting the system out of equilibrium and favoring the release of bound-protein from the structure with which it is interacting. The loss of bound protein is observed as a

loss of fluorescence from the intracellular structures. Because this change from bound to unbound is a first-order reaction, PARF kinetics can be fit to a single exponential decay, providing an apparent off-rate and half-life for loss of fluorescence. To test this approach, we compared PARF analysis of GFP, GFP-VASP, and GFP-Mito with previously published FRAP analyses of those constructs. FRAP exchange rates and PARF loss rates were similar. We then examined the PARF kinetics of a panel of actin-binding proteins in the presence and absence of jasplakinolide. As predicted, stabilization of the actin network resulted in slower loss of fluorescence. These results demonstrate that PARF analysis of non-equilibrium systems can reveal information about binding interactions without the infrastructure investment required for other quantitative fluorescence approaches.

P15

Board Number: B114

Quantitative screening of antifade mounting media for fluorescence microscopy.

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Despite advancements in dye chemistry, the development of brighter and more stable fluorescent proteins and advances in the sensitivity of microscopes, photobleaching remains a significant challenge in the capture and analysis of cellular images. Advanced imaging techniques such as three-dimensional Z-stacking require multiple images of the same section to be collected with intense illumination. Retention of fluorescent signal throughout the experiment is critical not only for image quality, but also quantification of the results. The mechanism of the photobleaching of fluorescent dyes is hypothesized to be via a photo-oxidative pathway, wherein the long-lived triplet excited state of a fluorescent molecule irreversibly complexes with reactive oxygen species. To mitigate the effects of photobleaching, “antifade” reagents are often added to mounting media. These reagents slow the loss of signal from fluorescent dyes during imaging experiments through several possible routes, including acting as reducing agents, reactive oxygen scavengers or triplet-state quenchers. A number of protocols exist for the preparation of mounting media, often glycerol and polyvinyl alcohol-based, containing reducing agents (e.g. para-phenylene diamine). In many cases, however, commercial mounting media provides decreased variability in performance over time required for careful cellular analysis. Choice of mounting medium is largely dependent upon the type of sample and sample preparation, the type of imaging involved and the specific fluorophores used. Often, choice of mounting media is a compromise of photobleach resistance, initial intensity, signal to background, compatibility with fluorophores, refractive index and other factors.

In order to evaluate antifade mounting media we have developed methods for quantitative assessment of photobleaching, initial intensity and signal-to-background using a combination of both confocal and wide field microscopy. Using these methods, we systematically screened a series of 20 fluorescent dyes, nuclear stains and fluorescent proteins (including TagGFP, TagRFP and mCherry) in multiple cell lines (HeLa, U2OS, A541). Samples were mounted using commonly used commercially-available mounting media for comparison. Ideally, mounting media should protect the broadest range of dye classes (e.g.

cyanine- or rhodamine-based) from photobleaching, while retaining bright initial intensity. Our analyses show that the performance of mounting media is highly variable and most will protect one subset of dyes significantly better than others, diminish initial intensity or increase off-cell background.

P16

Board Number: B115

Observation of Individual Secretory Granules in Living Mast Cells Using Confocal Microscopy.

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Mast cells, which are distributed in the mucous membranes and epithelial tissue of the body surface, cause various allergic symptoms by releasing inflammatory mediators such as histamine from their intracellular secretory granules (SGs) in response to allergic stimuli (degranulation). It is hoped that the development of treatments that inhibit degranulation will enable the mitigation of allergy symptoms. To do so, it is necessary to observe the intracellular dynamics of these SGs to elucidate the process of their maturation, intracellular transport, and degranulation. However, it has been difficult to observe individual and distinct SGs by the conventional microscopic methods (which is by tagging SG marker proteins with fluorescent proteins) since marked fluorescent dispersion makes the target outlines unclear. Here, to overcome this problem, we observed individual SGs by overexpressing green fluorescent protein (GFP) in the cytoplasm of mast cells and using confocal microscopy to photograph the “absence of GFP” representing SGs that arose as a result of GFP not penetrating the granular lumen. First, we demonstrated that the accuracy of this new method is higher than that of the conventional observation. To validate the principle, we observed polystyrene beads labeled with a red fluorescent dye in a GFP solution. When we constructed 3D images of the beads from sequential tomography images, the dispersion of light caused the images derived from the red fluorescence to be greatly distorted in the direction of the z-axis, while the images derived from the “absence of GFP” were spherical and corresponded to the theoretical volume of the beads. Next, in a similar manner, we overexpressed GFP in the cytoplasm of the rat mast cell line RBL-2H3 and constructed 3D images. From these images, we obtained information on the construction of individual SGs (number of particles, size, volume, and arrangement), as well as on the cell as a whole. We demonstrated that the cells in these images indeed showed SGs by co-localization of red fluorescent protein-fused SG marker proteins (Neuropeptide Y or VAMP7) co-expressed in the cells. Furthermore, by inducing degranulation, we obtained information on the structure of exocytotic SGs in the cells. In this presentation, based on the structural characteristics of the SGs that we have thus obtained, we will discuss the process of the maturation, intracellular transport, and degranulation of SGs.

P17**Board Number: B116****3D nano stiffness mapping of intracellular compartment using correlative light atomic force electron microscopy (CLAFEM).**M. Popoff¹, N. Barois², S. Janel², A. bongiovanni², E. Werkmeister², S. Divoux³, F. Perez³, F. Lafont¹;¹CNRS UMR 8204-CMIP, Institut Pasteur de Lille, Lille, France, ²BioImaging Center Lille, Institut Pasteur de Lille, Lille, France, ³CNRS UMR144, Institut Curie, Paris, France

Mechanical properties of biological material like cells and tissues are known to play a key role the cell differentiation, cell division, cancer, diseases and other processes. Atomic Force Microscopy is routinely used to gain access to the precise information on the sample's topography and physical properties. We propose here to unravel the elasticity of different identified organelles and microorganisms inside cells, at the nanoscale, with a novel technique combining stiffness mapping, super-resolution fluorescence and electronic microscopies, named CLAFEM (Correlative Light Atomic Electron Microscopies). We report herein the first results on bacteria, Golgi, actin tail comets and autophagosomes in both fixed and living cells.

P18**Board Number: B117****Continuous throughput and long-term observation of single-molecule FRET without immobilization.**S. Tyagi¹, V. Vandelinder², N. Banterle¹, G. Fuertes¹, S. Milles¹, M. Agez¹, E. Lemke¹;¹Structural and Computational Biology Unit, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany, ²Sandia National Laboratories, Albuquerque, NM

Single-molecule FRET (smFRET) studies through Total internal reflection fluorescence microscopy (TIRFM) is a powerful method to extract and reveal hidden heterogeneity in biological systems from long-term FRET trajectories of individual single molecules. TIRFM popularity also owes to the possibility of adding exogeneous reagent and following the biological processes over long-time. The high signal to noise (SNR) necessary for observing fluorescence from single emitters is achieved by tethering single molecules within the shallow evanescent field (less than 200 nm) generated at the interface of coverslip and buffer. This requirement of tethering molecules to coverslip restricts the applicability of TIRFM to a limited set of biomolecules as complex immobilization procedures cannot be generally applied and sometimes perturb the native state of the biological sample. Here we present a versatile technology, termed "SWIFT" (Single molecules Without Immobilization For TIRFM) that enables us to perform multisecond observation of freely diffusing single molecules with high SNR while bypassing the need for immobilization procedures. SWIFT is a microfluidic-nanofluidic hybrid platform where nanochannels are actively and reversibly generated by collapsing 1 µm deep flow channels using pressurized nitrogen gas in a control channel, which additionally increases photo stability of fluorophores by removing oxygen.

Biomolecules continuously flow through 12 parallel 100 nm deep channels, which keep them within the evanescent field. The microfluidic device is fabricated with soft lithography and molding techniques out of an elastomeric material, which enables to exploit the full potential of microfluidics including low-cost, ease of use and fast solution exchange. We show that several second long trajectories of thousands of molecules can be recorded with millisecond time resolution, illustrating the potential for long-term automated and high throughput experiments. We demonstrate the power of our method by studying a variety of complex nucleic acid and protein systems, including DNA, Holliday junctions, nucleosomes and human transglutaminase 2.

Reference:

Tyagi, S., Vandelinder, V., et al. Nature Methods (2014)

P19

Board Number: B118

Video-enhanced polychromatic polarized light microscope (VEP polscope): bringing colors to the colorless world.

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A conventional polarized light microscope, which is sometimes called a “polscope” for short, shows a two-dimensional image of birefringence due to structural or internal anisotropy of the specimen under investigation. The simplest polarizing setup, consisting of two crossed linear polars, produces pictures whose contrast is directionally sensitive. The image brightness varies proportionally with $\sin^2 2\alpha \sin^2(180^\circ - \delta/\lambda)$, where α is the specimen’s slow axis azimuth relatively to the direction of polarization of the illuminating light, δ is the specimen retardance and λ is the wavelength. If the slow axis and the light polarization are parallel, the brightness is zero. The brightness is highest if the slow axis is oriented at 45° and 135° respectively. Also the brightness is zero if the retardance equals to an integer number of wavelength. When the specimen under investigation is illuminated with white light and its retardance increases, then the brightness of all the wavelengths increases as well and the specimen appears grey. After the retardance has approached 400 nm, the blue part of the spectrum is suppressed and the specimen shifts to yellow and then red. Once the retardance reaches 600 nm, the red part of the spectrum has been blocked and the specimen turns blue and then green. The specimen exhibits Newton’s interference colors. In conventional polarized light microscopy, the color is solely determined by retardance and does not depend on the slow axis azimuth. The polarized light coloring is widely used in mineralogy and petrography. But this phenomenon could not be utilized in biology because most biological specimens exhibit a retardance of less than several tens of nanometers. Therefore, they are colorless. Even adding the first order red compensator, with a retardance of 575 nm does not help much. In this case, zero retardance corresponds to purple, and a slight color variation could be observed when the retardance change is at least 50 nm. Recently, we developed a new video-enhanced polychromatic polarized light microscope that produces interference colors at retardance

level of several nm. The polychromatic polscope employs a new principle of generating interference colors from white light. Here, color is determined by the orientation of the particle, not by its retardance. Thus, the full spectrum color can be achieved at a much lower retardance. The polychromatic polscope shows an orientation-independent birefringence image without requiring any digital computation. An eye or camera can directly see the colored polarization image in real time with a brightness corresponding to the retardance level and color corresponding to the slow axis azimuth. The previously colorless birefringent cell parts become vividly colored. We applied the new polychromatic polscope for imaging to a number of biological specimens including cancer tissue, malaria samples, mouse brain slices, various diatoms, zebrafish and sea urchins embryos, rotifer, paramecium, stentor, planaria, etc. Some of examples will be shown in the report.

P20

Board Number: B119

Improving the imaging of unstained specimens in bright field microscopy.

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Bright field is the simplest and most widespread light microscopy modality. However, its use in cellular biology has been limited due to lack of contrast in the imaging of thin, transparent samples such as cells. Instead, more evolved microscopy techniques (e. g. differential interference contrast, dark field, phase contrast, among others) have been used. An alternative to increase image contrast is deconvolution processing, a powerful method often used in fluorescence microscopy. However, application of deconvolution processing to bright field images has been scarce, mainly because acquisition of the corresponding point-spread function (PSF) has been difficult. In this work, we present direct measurement of the phase PSF of a high-aperture microscope operating in bright field. Polystyrene nanoparticles of 100 nm in diameter serve as the point objects that are imaged with high contrast and low noise using conventional microscopy plus digital image processing. To our knowledge, this is the first report that describes the experimental assessment of this function. The measured PSF allows us to demonstrate conventional deconvolution on the bright field images of living, unstained *Escherichia coli* cells, showing improved definition of cell boundaries and sub-cellular features. In addition, we were able to integrate the same digital imaging processing technique to an optical tweezers setup designed to perform single-molecule experiments. Using this setup, we were able to follow the stepping of the motor protein kinesin on clearly-imaged individual microtubules in real-time. In the context of optical manipulation, our microscopy approach using standard bright field eliminates the disadvantages of adding extra optical components that often constrain the properties of the optical trapping beam. Altogether, our studies provide a simple method to acquire the phase PSF, make possible the extension of deconvolution processing beyond fluorescence, and show that the improvement of bright field imaging via digital image processing can benefit single-molecule techniques such as optical tweezers.

P21**Board Number: B120****Exploring cellular ultrastructure in 3D by Array Tomography and SEM imaging: A case study on zebrafish immune cells isolated by FAC-sorting.**I.U. Wacker¹, C. Bartels¹, M. Thaler², C. Grabher³, R.R. Schroeder⁴;¹CAM/Cryo-EM, Universitaet Heidelberg, Heidelberg, Germany, ²Carl Zeiss Microscopy GmbH, Oberkochen, Germany, ³ITG, KIT, Karlsruhe, Germany, ⁴Cryo Electron Microscopy, Universitaet Heidelberg, Heidelberg, Germany

Array tomography (AT) has been introduced almost ten years ago, initially to characterize neurons by multiplexing antibody labeling on serial sections of brain slices adhering to glass slides (Micheva & Smith, 2007, *Neuron* 55, 26). Such arrays can also be imaged in a SEM when deposited on conductive substrates such as silicon wafers or metal-coated glass (Wacker & Schröder, 2013, *J Microsc*, 252, 93). Here AT is applied to unknown GFP-expressing populations of blood cells isolated from zebrafish by FAC-sorting. Contrary to human blood cells which can be identified and sorted after staining with different combinations of CD markers for zebrafish such an approach is not possible due to lack of suitable antibodies. Some blood cells, however, have typical ultrastructural features so we aimed at reconstructing whole cells in 3D. Sorted cell populations were chemically fixed and embedded in epoxide resin. Arrays of a few hundred serial sections were produced using an ultramicrotome and a custom-built handling device. Stacks of SEM images were recorded automatically with the newly developed Atlas-AT software (Carl Zeiss Microscopy), aligned and volume-rendered or segmented. We established organelle inventories of various cell populations of the lymphoid lineage isolated from three different hematopoietic organs: Whole kidney marrow (WKM), thymus, and spleen. We found two predominant phenotypes with similar organelle inventories but different surface morphologies. One phenotype, showing a ruffled surface represented the majority of cells in a sort from spleen, was present to a lesser degree in WKM and not at all in thymus. The other phenotype had a smooth surface and few longer processes, constituted the major population in the sort from thymus and proportionally smaller populations in spleen and WKM. These observations were confirmed quantitatively by classifying and counting all cell profiles present in one section – on several sections of an array far enough apart to ensure that no cell was counted twice. AT can also be used to identify rare events: In co-cultures of cell populations isolated from WKM or thymus with the human promyelocytic leukemia cell line HL60 we found a few cell couples consisting of fish cells attached to HL60 cells. Due to the close contact between the plasmamembranes of both cells and a concentration of secretory organelles towards the contact zone we concluded this to be immunological synapses, indicating cytotoxic activity for our unknown cells. This was proven by time-lapse light microscopy imaging of co-cultures. Taken together our case study demonstrates that AT can be extended from brain mapping to general imaging of exceedingly large volumes, such as whole cells, tissues and even small organisms at ultrastructural resolution.

P22**Board Number: B121****Measuring the distribution of taurine molecule inside biological tissue via intrinsic molecular vibrations using nonlinear Raman spectroscopy.**M. Kawagishi¹, Y. Obara², T. Suzuki², M. Hayashi³, K. Misawa², S. Terada¹;¹Dept Neuroanat Cell Neurobiol, Grad Sch Med Dent Sci, Tokyo Med Dent Univ (TMDU), Bunkyo, Tokyo, Japan, ²Dept Applied Physics, Tokyo Univ Agricul Technol (TUAT), Koganei, Tokyo, Japan, ³Wired Co., Ltd., Komae, Tokyo, Japan

Distributions of small organic molecule (less than 300 Da) compounds inside biological tissue have been obscure because of the lack of appropriate methods to measure them. Although fluorescence techniques are widely used to characterize the localization of large molecules such as proteins and nucleic acids, they cannot be easily applied to small molecule compounds: Fluorescent labels are relatively large compared to the target compounds and can change the properties of them. Raman spectroscopy is a technique to study vibrational information intrinsic to and characteristic of the chemical species of compounds. We used coherent anti-Stokes Raman scattering (CARS) spectroscopy to detect and identify a small molecule compound, taurine, without labeling. Molecular species could be uniquely identified from the spectral shape of the broadband vibrational spectra of target molecule. The local distribution of target molecule could be determined from the spectral intensity. We have developed a phase-sensitive CARS spectroscopy capable of simultaneous measurement of the broadband spectrum while maintaining a high frequency resolution. We utilized this technique to measure taurine inside mouse cornea tissue soaked in solution. We detected a Raman peak of taurine near 1000 wavenumber / cm inside cornea, and successfully characterized its depth profile in the tissue. Our CARS spectra measurement can be a promising method to measure and visualize the distribution of small bio-related compounds in biological background without using any labeling, paving the way for new cell biological analysis in various disciplines.

P23**Board Number: B122****In vivo tracking of label-free resident cells in murine tissues via third harmonic generation microscopy.**D. Malide¹;¹Light microscopy core facility, NIH/NHLBI, Bethesda, MD

Although fluorescent compounds and proteins have emerged as invaluable tools to mark cells and structures of interest for confocal and two-photon microscopy imaging, such probes can interfere with cellular functions. While it is possible to visualize naturally occurring signals via two-photon microscopy, imaging remains challenging and has been limited primarily to thin and transparent biological samples. We developed and validated a methodology for high resolution 3D harmonic imaging of intact murine

tissues that requires no fluorescent labeling, based on epi- third-harmonic generation (THG) imaging by pulsed near-infrared light in the 1200–1300 nm range. Visualizing THG in the epi- (backscattered) direction enabled high resolution depth-resolved imaging of whole mount, intact tissues without the need for physical sectioning. We demonstrated intrinsic THG interface signals, outlining cell membranes and tissue inhomogeneities, revealing subcellular resolution of 3D architecture in various murine tissues and organs. These included subcutaneous and perivisceral white and brown adipose tissue, with clearly demarcated lipid droplets, blood vessels and cells, and myelinated peripheral nerves. We also visually dissected intracellular lipid-rich granules in adrenal glands, mammary glands, sebaceous glands, pancreas, liver, and spleen. Characteristic patterns were also observed in the skeletal muscle, heart, bone, skin, lung, brain, and spinal cord. Using this methodology, we examined the intracellular lipid deposits in healthy or diseased mouse tissues, including fatty liver, ischemic cardiomyocytes, and in the atherosclerotic aortic wall. Moreover, multiharmonic, combined THG and second harmonic generation (SHG) images can be assessed qualitatively and quantitatively to evaluate 3D distribution of the cells in label-free tissues and organs in control and experimental conditions. In addition, we demonstrated the feasibility of multimodal imaging by combining simultaneously excited harmonic signals with fluorescence-excitation of red-shifted fluorescent proteins expressed in vivo. Finally, time-lapse 3D THG imaging, obtained with faster acquisition settings, enabled dynamic cellular tracking in intact undisturbed tissues. This methodology uses commercially available turn-key systems allowing easy implementation of a versatile tool to track resident label-free cells in their undisturbed surroundings, as a powerful alternative or complementary contrast mechanism to fluorescence-based techniques. THG imaging could especially facilitate development of noninvasive approaches for optical biopsies of various tissue-types in diagnostic medicine.

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Board Number: B123

Molecular interrogation of single-cells using ion mobility separation mass spectrometry.

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Mass spectrometry (MS) with liquid chromatography (LC) is a standard analytical tool for analyzing small molecules in biological samples. Reduction of ion suppression due chromatography separation enhances detection of molecules. It also provides retention time values to increase identification confidence of molecules. However, due to their limited volume, analysis of single-cells with traditional chromatography methods is unviable. To overcome this limitation, we performed molecular analysis of single-cells using an on-the-fly approach, ion mobility separation (IMS). In situ sampling of cellular contents was achieved by laser ablation (laser ablation electrospray ionization or LAESI)¹ or capillary microsampling² prior to electrospray ionization. For this study, pollen tubes (*Lilium longiflorum*) and

hepatocyte (*Homo sapiens*) were used as model plant and animal cells, respectively. IMS improved signal-to-noise ratios of the ions from single-cells by discriminating them from isobaric ones found in the background. This led to an increase in the total number of molecular species detected from single-cells. In addition, the rotationally-averaged collision cross-sections (CCS) of ions were obtained from their drift times during IMS. The measured CCS of ions were used as additional parameters to increase the identification confidence of molecules from single-cells in combination with accurate mass-to-charge ratios and isotope distribution patterns. The reference CCS values were primarily obtained from existing literature and experiments with standards. When experimental values were unavailable, approximate CCS values were generated *in silico*. The identification of molecules of interest in single-cells was further confirmed by structural elucidation using tandem MS of the respective ions in bulk cell populations.

References:

- [1] B. Shrestha and A. Vertes, In Situ Metabolic Profiling of Single Cells by Laser Ablation Electrospray Ionization Mass Spectrometry. *Analytical Chemistry*, 2009, 81, (20), 8265-8271
- [2] L. Zhang, D. P. Foreman, P. A. Grant, B. Shrestha, S. A. Moody, F. Villiers, J. M. Kwak, and A. Vertes, In Situ Metabolic Analysis of Single Plant Cells by Capillary Microsampling and Electrospray Ionization Mass Spectrometry with Ion Mobility Separation. *Analyst*, 2014, July (online)

P25

Board Number: B124

DEVELOPMENT OF CAPILLARY BASED VACUUM ASSISTED INSTRUMENTS FOR RAPID AND EFFICIENT COLLECTION OF SINGLE CELLS FROM CELL CULTURES AND MICRODISSECTION OF HETEROGENEOUS TISSUES.

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Cell specific studies are imperative for sound research in basic and translational cell biology. Collecting single or individual cells directly from cell cultures for downstream single cell analysis or recultivation purposes has remained challenging. Similarly, tissue heterogeneity poses significant challenge for retrieval of cell and region specific information. Existing approaches, such as flow sorting and laser assisted acquisition, are costly, sophisticated and often methodologically limited. We have developed two capillary-based vacuum-assisted cell and tissue acquisition instruments (Kuiqpick™ and Unipick™) with which individual fluorescently labeled and/or morphologically distinct cells can be acquired from any cultures grown in standard cell culture dishes. Moreover, the instruments are suitable for individual cell collection and region specific acquisition of cell clusters and subanatomical areas from complex heterogeneous tissues such as the brain. While Kuiqpick™ is integrated with a fluorescence compatible inverted microscope (Olympus CKX41), Unipick™ is a free standing instrument that may be mounted over any inverted microscope, providing additional flexibility in the laboratory. Both instruments demonstrate a wide range of cell and tissue acquisition parameters. They permit rapid collection of

individual cells from various cell cultures for various downstream applications including single cell analyses. Because small sample volume is incremental for single cell research, Kuiqpick/Unipick enables the collection of a single cell in as small as 12 nl. The current work demonstrates the efficiency of Kuiqpick™ for cell collection from adherent, suspended, and 3D cultures. Individual cells were collected from primary neural progenitor (NPC), SH-SY5Y, CHO and MDA-MB-435 cell cultures based on morphology or fluorescent label. Further, the collected cells were successfully recultured, demonstrating the minimal effect the process has on cellular viability (up to 95%). The clonal expansion of collected single SH-SY5Y and CHO cells was demonstrated within 6 and 25 days, respectively. In addition, applicability of the instruments for the collection of single cells grown in three-dimensional (3D) culture system was shown using MDA-MB-435 cells. To further demonstrate Kuiqpick/Unipick's capabilities for cell and region acquisition from complex heterogeneous tissues, single Purkinje cells and subanatomical regions (e.g. CA1-3, dentate gyrus) were efficiently collected from rat brain tissue sections. The instrument dissected brain sections with thickness from 10 to 500 μm. Native, fresh frozen, and sucrose treated tissues could be used. High quality RNA and proteins were isolated from the collected samples.

P26

Board Number: B125

Development of single strand DNA aptamer for cell surface of human umbilical vein endothelial cell.

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Aptamer is a stranded molecular consisted of nucleic acid or amino acid that works as a ligand binding to targets such as protein, metal and organic small molecular. Aptamer is made by "Systematic Evolution of Ligands by EXponential enrichment" (SELEX) method from huge number of aptamers by repeating selection procedure. Aptamer is more useful than antibody for cell labeling and tissue engineering because it is easy to elaborate clones stable and be modified with genetic engineering procedures such as adding fluorescent material or biotin, or recombining the sequence to stronger affinity ligand. Furthermore, since aptamer is degradable by nuclease, it is easy to detach the aptamer labeling. Therefore, labeled cells with aptamer to perform fluorescence-activated cell sorting (FACS) or Magnetic-activated cell sorting (MACS) method is undoable to intact condition with nuclease, while antibody can't it. SELEX method can be applied to cell surface targets or tissue. This method is called Cell-SELEX and can also make aptamer against unknown targets of cell surface. Human umbilical vein endothelial cell is used for physiological, pharmacological investigations and tissue engineering. Therefore, aptamer against cell surface of HUVEC will be useful for these research fields. ssDNA aptamer library contained a central randomized sequence of 40 nucleotides flanked by primer hybridization sites. ssDNA aptamers against cell surface of HUVEC was obtained by a modified cell-SELEX method. In briefly, after aptamer library was incubated with cells, the unbound aptamer removed, and then, the bounded ssDNA was

exfoliated from cell surface by trypsinization not to leak cytosol materials of cell. After that, bound ssDNA was amplified by the PCR and ssDNA was made by alkaline treatment from dsDNA as the next round template. Totally 10 rounds of selection were performed. The selected aptamer sequences were read by a next generation sequence and classified the sequence by a bioinformatics. Each round aptamer was labeled with FITC or biotin, and binding affinity was analyzed by MACS or a fluorescence microscopy. In results, almost of HUVEC was collected by the selected aptamer-magnetic beads conjugates. Moreover, the affinity was increased with repeated selection. Moreover, FITC-labeled aptamer stained the surface of the cells. These results indicated that our modified cell-SELEX method allowed us to make ssDNA aptamer against cell surface of HUVEC as a ligand. Aptamer for HUVEC is expected to use for cell sorting, micropattern of cell, and many biotechniques. Furthermore, reading sequence of selected aptamers in full detail by next generation sequence method gives us many information and it is possible to select the sequence against the target cells even if there are a wide variety of cell types in same tube.

P27

Board Number: B126

Reconstructing alveolar epithelial development using single-cell RNA-seq.

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Although aspects of alveolar development have been studied by marker expression analysis and fate-mapping, the mechanisms that control the progression of lung progenitors along distinct lineages into mature alveolar cell types remain incompletely known, in part because of the limited number of lineage markers and the effects of ensemble averaging in conventional transcriptome analysis experiments on cell populations. With single-cell transcriptome analysis we were able to directly measure the various cell types and hierarchies in the developing lung. We used microfluidic single-cell RNA sequencing (RNA-seq) on 198 individual cells at four different stages encompassing alveolar differentiation to measure the transcriptional states which define the developmental and cellular hierarchy of the distal mouse lung epithelium. We empirically classified cells into distinct groups by using an unbiased genome-wide approach that did not require a priori knowledge of the underlying cell types or the previous purification of cell populations. The results confirmed the basic outlines of the classical model of epithelial cell-type diversity in the distal lung and led to the discovery of many previously unknown cell-type markers, including transcriptional regulators that discriminate between the different populations. We reconstructed the molecular steps during maturation of bipotential progenitors along both alveolar lineages and elucidated the full life cycle of the alveolar type 2 cell lineage. This single-cell genomics

approach is applicable to any developing or mature tissue to robustly delineate molecularly distinct cell types, define progenitors and lineage hierarchies, and identify lineage-specific regulatory factors.

P28

Board Number: B127

Application of a live-cell RNA detection technology for use in cellular High Throughput Screening assays.

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High-throughput screening (HTS) approaches enable the systematic generation of vast amounts of experimental data through the automated processing and analysis of large numbers of biological samples¹. Many traditional biological experiments have been expanded and modified to take advantage of HTS technologies. Modern platforms contain internal systems to automatically control incubation temperature, exchange growth media, and maintain pH levels in sample plates, allowing researchers to perform large scale studies without compromising their demand for biological relevance. Gene expression analysis is an area of research that is widely pursued today using HTS approaches. Specifically, several HTS techniques exist today for the large-scale identification and analysis of RNA content, such as in the context of identifying gene responses to drug compounds. However, many of these techniques require cell lysis for subsequent PCR amplification steps, which compromises cell viability and integrity, and prohibits the use of the assayed cells for downstream characterization and functional studies. Further, these assays also lack the ability to discern gene expression differences within heterogeneous cell samples. Performing direct RNA detection in live cells would address these key issues, enabling the researcher to gain even more biologically relevant information from critical HTS experiments. Retaining overall cell viability and integrity yields more meaningful biological data. In addition, single-cell gene expression analysis is made possible when resolution of heterogeneous populations is needed. This approach also enables the use of the interrogated cells for further downstream functional assays. In this poster, we demonstrate the live-cell detection of ERBB2 mRNA using SmartFlare™ RNA detection probes in two breast cancer cell lines, which differ in their expression of this therapeutic marker and exhibit differential responses to drug treatments. These cells were cocultured in varying ratios, incubated with SmartFlare™ probes in normal culture conditions, and directly assayed for ERBB2 mRNA. We show that sensitivity in detection can be achieved at the single-cell level, enabling greater understanding of the inherent heterogeneity within mixed cell samples, while retaining the scalability and throughput commensurate with HTS approaches. Live cell RNA detection enables single-cell analysis, preserves cellular viability and integrity, and allows samples to be further utilized for downstream research. Researchers who seek to maximize data output, minimize sample preparation, and generate biologically-relevant data from HTS gene expression experiments can benefit from incorporating live cell RNA detection into their workflows.

P29**Board Number: B128****An automatable 3-dimensional cell invasion assay compatible with high content analysis.**J. Fronczak¹, S.I. Montanez-Sauri¹, M.S. Gajeski¹, B.R. Mader¹, T.G. Burland²;¹Platypus Technologies, LLC, Madison, WI, ²Platypus Technologies LLC, Madison, WI

Cell invasion is the movement of cells through extracellular matrix (ECM). Invasion is of particular concern in cancer, where invading tumor cells lead to metastasis, the deadliest aspect of the disease. High-throughput assays suitable for screening the impact of candidate anti-cancer drugs on cell invasion are therefore highly sought after. Traditionally, cell invasion is quantified through “transwell” assays in which movement of cells from one chamber to another chamber through a synthetic membrane and ECM layer is monitored. However, transwell assays do not readily facilitate automation or real-time monitoring of invasion, nor simultaneous microscopic analysis of cell phenotypes such as changes in overall morphology, nuclear structure, etc. Moreover, the use of a synthetic membrane to separate the two transwell chambers poorly simulates conditions found in vivo. Here we report development of a fully automatable cell invasion assay that is configured in an industry-standard 96-well format and that can be used to monitor cell movement in three dimensions through ECM. The new assay seeds cells around an “exclusion zone”. Following cell attachment, an overlay of collagen I is added. Cell movement into the exclusion zone is then monitored in real-time, and cell morphology and physiology may also be monitored simultaneously. We report data comparing the information content and multiplex capabilities of traditional transwell invasion assays with the new exclusion zone invasion assay. We conclude that the fully automatable invasion assay presented in this work delivers major improvements in efficiency and information content over transwell assays, and promises to facilitate high content screening of cancer drug candidates for their potential to inhibit metastasis.

P30**Board Number: B129****HC StratoMineR: A web-based data mining tool for the rapid analysis of high content data sets.**

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High content screening combines high throughput screening, and automated fluorescence microscopy and image analysis. The field has long been supported by mature technologies for automated liquid handling and automated image acquisition and analysis, such that large scale high content screens are feasible. There are however, few tools available to aid in the analysis of the large multiparameter data sets that are produced by these screens.

We have developed a web-based tool called HC StratoMineR to aid the mining of high content screening datasets. This is a decision supportive data pipelining tool that assists even non-expert users in the efficient analysis of high content data sets. Our goal was to generate easy-to-read visualizations that make it possible to compare cellular morphological responses. Visualizations and other information provided are useful for the selection of useful parameters that have been generated by image analysis software. HC StratoMineR assists in the quality control of the screen and allows for the generation of a hit list based on cellular morphology. These hits are then classified using multivariate analysis techniques. Critically HC StratoMineR allows for a final report, where all decisions made are saved so that the next experiment can be analysed using the same methods.

We have validated HC StratoMineR using chemical and RNAi knock-down screens. Our work shows that HC StratoMineR greatly enhances and accelerates the analysis of high content data sets. We show that the software does indeed segregate samples according to the cellular morphology they induce. We demonstrate that HC StratoMineR can highlight structure-morphology relationships in compound screens and gene function-morphology relationships in RNAi screens. This allows researchers to extract the greatest benefit from high content data sets.

P31**Board Number: B130****Lipid nanoparticle delivery of RNA for loss-of-function and gain-of-function studies in primary neurons in vitro and in vivo.**D. Zwaenepoel¹, A. S. Ansari¹, A. Thomas¹, C. Walsh¹, T. Leaver¹, A. Wild¹, K. Ou¹, J. Taylor¹, E. Ramsay¹;¹Precision Nanosystems Inc., Vancouver, BC

A lipid nanoparticle (LNP) technology was developed to deliver mRNA and siRNA into neurons in vitro and in vivo with high efficiency and low toxicity. LNPs encapsulating RNA were prepared using the NanoAssemblr microfluidics platform. LNPs mimic low-density-lipoproteins (LDL) which are taken up by cells through the LDL-receptor in presence of Apolipoprotein E4.

In vitro - More than 95% of Primary E18 rat Cortical Neurons (13 days in vitro) mixed with glial cells (PCN) incubated 4h with siRNA-LNP (100 ng/ml) showed fluorescent LNP uptake (Flow Cytometry, Confocal Microscopy). PCN incubated with 100 ng/mL of PTEN siRNA-LNP (siPTEN-LNP) for 72h showed > 90% knockdown (RT-qPCR, Western Blot). PCN incubated with HPRT siRNA-LNP (siHPRT-LNP) showed sustained knockdown of > 70% for 21 days after a single dose treatment (100 ng/mL) on Day 0 (RT-qPCR). PCN treated with 100 ng/mL siPTEN-LNP alone, or 100 ng/mL siHPRT-LNP alone, or a combination of both nanoparticle preparations showed respective knockdown of PTEN and HPRT 72 hours post treatment (RT-qPCR). Comparison of Neuro9Kit to contemporary transfection kits showed significantly higher knockdown efficiency with 100 ng/mL sPTEN-LNP 72h post treatment (qPCR). Neuro9Kit showed no toxicity at 100 ng/ml siRNA-LNP 72 hours post treatment (LDH).

PCN incubated with 500 ng/mL GFP mRNA-LNP and/or 500 ng/ml mCherry mRNA-LNP for 72h showed GFP and/or mCherry expression (Flow cytometry, Confocal Microscopy).

In vivo - A somatosensory injection of siPTEN-LNP (500 nL at 5 mg/mL for 10 minutes) in Spargue-Dawley rats showed > 80 % knockdown in cortical slices up to 1 mm from the injection site 5 days post-injection (Western Blot).

Solid-core LNP manufactured using the NanoAssemblr demonstrated rapid uptake by neurons which mediated effective and sustained silencing/expression of a target genes in vitro and in vivo with no detectable toxicity. This technology offers a simple and flexible alternative to viral vectors, electroporation and lipofection for loss- and gain-of-function studies in neural development, injury and degeneration.

P32**Board Number: B131****Super-active TALEN with improved stability at 37 degree Celsius enables highly efficient and homogeneous gene knockout in mammalian embryos.**K. Ikeda¹, Y. Terahara¹, K. Sumiyama¹, N. Miyashita¹, Y. Okada¹;¹RIKEN Quantitative Biology Center (QBIC), Osaka, Japan

Gene editing *in vivo* has become possible by the development of artificial nucleases that can be designed to cut the genome DNA selectively at the target site in the genome. The DNA binding domain of a plant pathogen protein called Transcription Activator-Like Effector (TALE) has been used as the designable sequence specific DNA-binding domain. The fusion protein of TALE and the nuclease domain of Fok I endonuclease, called TALE nuclease or TALEN, has been proven to be useful for the genome editing in lower vertebrates such as zebrafish and *Xenopus*. In mammalian cells and embryos, however, TALEN often shows poor activity, which limited its applications. We have surmised that the TALE protein might not be stable at 37 °C, because the physiological temperature for the plant pathogen is around 25 °C. Through the analysis of the crystal structure of TALE protein, we have chosen two amino acid residues that might be pivotal for the stable alignment of the linear solenoid structure of TALE. Several mutations were introduced, and some successfully showed significantly higher activity at 37 °C both *in vitro* and *in vivo*. All-atom molecular dynamics simulations confirmed the stabilization of the conformation. The mutated residues apparently suppressed the intramolecular fluctuations. We have, therefore, named this mutant TALEN as “super-active” TALEN and examined its activity in mouse embryos. We have designed TALENs, both wild type and super-active mutant, for the mouse tyrosinase gene. The mRNAs for the TALENs were introduced to the pronuclear stage mouse embryos. The wild type TALENs failed to introduce mutations, and the coat color of the babies were agouti. Contrastingly, more than half of the babies were albino with super-active TALENs, which means both alleles of the tyrosinase gene were mutated throughout the body. Interestingly, CRISPR/Cas9 mediated gene editing of tyrosinase gene in mouse pronuclear stage embryo yielded even higher rate of coat color mutants. However, the babies were mostly mosaic of agouti and white, indicating that only some population of cells got biallelic mutation. Consistently, the genome analyses detected only less than four different alleles in each baby with super-active TALEN, while more than eight alleles were often detected in a single CRISPR/Cas9 baby. These results suggest that super-active TALEN shows its activity at two-cell stage, earlier than CRISPR/Cas9, suggesting that super-active TALEN might serve as an effective tool for the genome editing in mammalian cells and embryos.

P33**Board Number: B132****Establishment of an in vivo electroporation method into postnatal newborn neurons in the dentate gyrus.**H. Ito¹, R. Morishita¹, I. Ikuko¹, K. Nagata¹;¹Mol Neurobiol, Inst Developmental Res/Aichi Human Service Ctr, Kasugai, Japan

Electroporation-mediated gene transfer has been developed for the analysis of mammalian brain development in vivo. Indeed, in utero electroporation method is widely used for the investigation of the mouse embryonic cortical development while in vivo electroporation using neonatal mouse brain is employed for the analysis of the rostral migratory stream (RMS) and postnatal olfactory neurogenesis. In the present study, we established a stable gene-transfer method to dentate gyrus (DG) neurons by carefully determining the in vivo electroporation conditions, such as position and direction of electrode, voltage for electric pulses, and interval between electroporation and sample preparation. Consequently, GFP-positive cells in DG were observed to extend branched dendrites and long axons into the molecular layer and the hilus, respectively, 21 days after electroporation. They were morphologically identified as dentate granule neurons with many protrusions on dendrites, and some of them had wide head and thin neck that resembled matured mushroom spines. Expression of GFP in dentate neurons sustained for at least 9 months after electroporation under our experimental conditions. Taken together, the method developed here could be a powerful new tool for the analysis of the postnatal DG development.

P34**Board Number: B133****Development of Transgenic Chickens for Studies on Retinoic Acid Signaling.**J. Summers¹;¹Department of Cell Biology, University of Oklahoma Health Science Center, Oklahoma City, OK

Introduction. Despite the significance of retinoic acid (atRA) in the growth and development of many major organ systems, visualization of endogenous atRA has only been indirectly determined. Moreover, due to the fundamental importance of atRA in early development, studies aimed at understanding the function of atRA in late embryogenesis or postnatal development using conventional genetic approaches have been severely limited. Therefore, the goal of this project is to develop two lines of germline transgenic chickens that will allow, for the first time, visualization and modulation of endogenous atRA in embryonic and post-hatch chicks. **Methods.** The pminiTol2 backbone was used to generate two constructs in order to 1) visualize endogenous atRA gradients (GEPRA B) and 2) conditionally lower endogenous atRA concentrations (tet-RALDH2 shRNA). The GEPRA B construct was cloned into the pminiTol2 plasmid containing a pCAGGS promoter for optimal expression in the chicken. The tet-RALDH2 shRNA construct was modified from a third generation lentiviral vector, the pINDUCER series, that allows tetracycline-inducible knockdown of RALDH2 as well as the expression of eGFP and DS

red fluorescent proteins to monitor transfection and induction, respectively, using a single vector. The minitol2 transposon system was used to deliver each of these constructs to circulating primordial germ cells (PGCs) in chick embryos through a recently developed direct injection technique. Briefly, transfection complexes consisting of 0.6 ug of pMiniTol-GEPRA or tet-RALDH2 shRNA, 1.2 ug of a plasmid containing the transposase sequence under the control of the CMV IE promoter, and Lipfectamine 2000 were prepared. Chick embryos stage 14 – 16 HH (~72 – 74 hrs incubation) were injected with 1 – 2 ul of the transfection complex using a pulled glass capillary micropipette inserted into the caudal portion of the left dorsal aorta. Eggs were sealed with surgical tape and incubated normally. To estimate transfection efficiency, chick embryo gonads were dissected and examined for eGFP or YFP fluorescence at various stages. To confirm transfection of PGC's, gonads were immunolabeled using antibodies against the stem cell antigen, SSEA-1. **Results.** The tet-RALDH2 shRNA vector significantly reduced RALDH2 protein expression in vitro. Embryos between days 7 and 14 of incubation expressed eGFP (for tet-RALDH2 shRNA injected embryos) or YFP (for GEPRA injected embryos), preferentially in the gonads. Co-localization of YFP or eGFP with SSEA-1 confirmed the presence of transgenic PGC's in embryonic gonads. **Conclusions.** This approach provides a promising alternative to the use of standard transgenic model organisms, and will enable, for the first time, functional studies on retinoic acid signaling in embryonic and post-hatch chickens.

P35

Board Number: B134

Functional Annotation for TCA and DNA Repair for Insects and Plants: A Survey.

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In the area of agriculture, plants and insects work in harmony or against each other to provide nourishment for the rest of the animal kingdom. These species are being studied by numerous scientists, hence it would be of deep interest to determine how well these works are collected, annotated and stored. Thus, a survey of functional annotations for agriculturally important plants and Insects was performed. We focused on two of the most common metabolic pathways amongst species, the Tricarboxylic Acid (TCA) Cycle and DNA Repair. The TCA cycle uses a sequence of chemical reactions to generate energy through the oxidation of acetate from carbohydrates, proteins and fats into carbon dioxide. DNA can be damaged by endogenous and exogenous agents, causing mutation and other serious conditions. DNA Repair is a series of enzymatic processes that correct the lethal changes that affect the sequence of a DNA molecule. We selected 39 Insects, 64 Plants and used QuickGO to find all annotations associated with each species using a taxonomy ID. Then, we filtered the annotations by using the GO Terms for the TCA cycle (GO:0006099) & DNA Repair (GO:0006281). Our results indicated that sixty-three percent of the selected species had annotations that were associated with the TCA cycle

or DNA Repair. Twenty-two percent of the selected species had annotations that were associated with both DNA Repair and the TCA cycle. Therefore, we conclude that more annotations of these biological processes species needs to be done.

P36

Board Number: B135

Wild and Domestic Animals Surveyed for Functional Annotations of the TCA Cycle and Apoptosis.

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There is so much information from biological processes of all organisms that needs to be stored by bioinformatics databases. The European Biological Institute (EBI) hosts one of the databases that are used to store annotated information on all organisms. With this study, we focused on two very important biological processes, the Tricarboxylic Acid cycle (TCA) and the Apoptotic process. The TCA cycle uses a series of chemical reactions to generate energy through aerobic respiration. Apoptosis, described as cell suicide or programmed cell death, is essential to maintain appropriate cell numbers in organisms. Our objective was to determine the number of animals, both wild and domesticated, which the TCA Cycle and Apoptosis are annotated. It is hypothesized that all organisms studied will have annotations for these two very important biological pathways the TCA cycle and Apoptosis. On the EBI database, we used QuickGO to retrieve all annotations associated with the taxonomy ID of the selected animals. The annotation was then filtered by using the GO term for the Krebs cycle (GO:0006099) and Apoptotic process (GO:0006915). Out of 13 wild animals studied only 5 had annotations for the TCA cycle and Apoptosis. Only 4 out 19 domesticated animals had annotations for Apoptosis, while none of 19 had annotations for the TCA cycle alone. Our data indicate that more work needs to be done by biocurators for annotations of the TCA cycle and Apoptosis in wild and domesticated animals.

P37**Board Number: B136****Microsecond-Pulsed Dielectric Barrier Discharge Plasma Treatment Promotes Murine Ear Regeneration.**N. Chernets¹, D.S. Kurpad¹, Q. Zhang¹, Y. Song¹, T.A. Freeman²;¹Orthopaedic Surgery, Thomas Jefferson University, Philadelphia, PA, ²Orthopaedic Surgery, Thomas Jefferson University, Philadelphia, PA

The ability of mammals to regenerate damaged tissue is limited [1-3], and more commonly wound repair results in scar tissue formation. In this report, we describe the application of non-thermal atmospheric dielectric barrier discharge plasma (DBD-plasma) as a novel modality which can promote regeneration of new ear tissue in a non-regenerating mouse's ear after injury. It has been shown that intracellular reactive oxygen species (ROS) are elevated in response to DBD-plasma [4]. In our recent study of DBD-plasma treatment of mouse autopods, we reported that DBD-plasma generated ROS to activate signaling pathways and induced an increase of wnt activation in the distal apical epidermal ridge (AER) to enhance digit growth and elongation [4]. As parallels exist between the functionality of the AER in autopod development and the blastema in regeneration, we hypothesized that DBD-plasma could be applied to the wounded ear to induce enhanced epimorphic regeneration. To determine if DBD-plasma could promote regeneration, a through-and-through circular hole was punched in the center of the cartilaginous part the ear of non-regenerative C67BL6 mice using a 2 mm biopsy punch. A single 5 sec treatment of DBD-plasma each day for 5 days after the punch to one ear, while the other punched ear remained as an untreated control. Images of the ear holes were acquired for 32 days and the area of the hole was determined using image analysis. Histological staining and immunohistochemistry of ear regeneration samples were used to evaluate the structure of the wound epithelium and to determine the extent of tissue regeneration. DBD-plasma treated mouse ears showed accelerated wound closure, formation of blastema-like structures and replacement of cartilage, hair follicles and sebaceous glands as compared to bilateral controls. DBD-plasma treated mouse ears had a significantly higher closure rate on the 32nd day as compared to control ears (n=10, p

P38**Board Number: B137****Electrostimulation of Human Neural Stem Cells to Enhance Neuronal Differentiation.**F. Ghasemi Tahrir¹, G. Jin¹, W. Ma¹, W.H. Suh¹;¹Bioengineering, Temple University, Philadelphia, PA

Differentiation guiding techniques (chemical and non-chemical, all included) for directing the differentiation of stem cells (to a specific phenotype) are considered essential platform technologies that can one-day aid in the development of clinical treatments associated with nervous system diseases

(i.e., Alzheimer's disease, Parkinson's disease, spinal cord injury). In the present work, electrostimulation, in particular, has been utilized as an enabling technology that promoted the differentiation of neural stem cells to neurons. We will talk about a custom-designed current stimulation cell culture device that enhanced neuronal differentiation of human adult neural stem cells (hANSCs). The differentiation via electrostimulation was confirmed by live-cell (fluorescence and confocal) microscopy, immunocytochemistry (ICC) and reverse transcription quantitative PCR (RT-qPCR). Our results indicated that precisely controlled electrostimulation over few weeks significantly increased the expression of Tubulin beta-3, which is a neuronal marker.

P39

Board Number: B138

Kinetics of Intracellular Nitric Oxide Production and Cytotoxicity in Lipopolysaccharide and Inteferon- γ -Induced Macrophage Activation.

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Nitric oxide is a multi-functional biomolecule that can function as a bactericidal or tumoricidal mediator. The generation of nitric oxide plays a key role in several processes such as cellular proliferation, metastasis and apoptosis and has been suggested to modulate both tumorigenesis and tumor killing depending on its concentration, cell type and the oxidative milieu. It is well established that bacterial lipopolysaccharides (LPS) and interferon (IFN)- γ are potent inducers of nitric oxide production in murine macrophages which may contribute significantly to host defense mechanism against microbial pathogens. In this study we have systematically evaluated the mechanism and kinetics of nitric oxide production and subsequent cytotoxicity on lipopolysaccharide (LPS) and IFN- γ treatment of murine macrophage RAW264.7 cells. The cells were analyzed after treatment with a series of novel simplified assays on a compact microcapillary based flowcytometer including assay for intracellular nitric oxide detection, apoptotic assay for annexin V based detection along with cell death, caspase-3/7 activity and mitochondrial membrane depolarization. The kinetic studies clearly demonstrate the early production of nitric oxide observed at 6 hours and sustained levels through 48 hours of treatment. While annexin V based apoptosis and cell death were increasingly observed after 12 hours of treatment, no mitochondrial depolarization was observed throughout. Evaluation of caspase-3/7 activity in LPS-IFN γ treated cells showed a moderate caspase-3/7 activity after 6 hours and saturated at 24 hours with only 50% of the cells displaying caspase-3/7 activation which is another distinctive indicator of apoptosis. Our data suggest that nitric oxide production and subsequent NO-mediated induction of apoptosis proceeds through alternative cell death pathways that do not involve mitochondrial depolarization. These results thus provide interesting insights into the mechanism of cytotoxicity for intracellular nitric oxide production and allows for understanding characteristic of lipopolysaccharide and IFN γ -induced apoptosis of macrophage RAW264.7 cells.

P40**Board Number: B139****Directly observed membrane disruption and resealing during centrifugation of sea urchin eggs.**K. Miyake¹, M. Goda², S. Inoue³, N. Araki¹;¹Dep.Hist.Cell.Biol., Kagawa University, Faculty of Medicine, Kita-gun, Kagawa, Japan, ²Nagoya Univ, CeSPI, Nagoya, Japan, ³Marine Biological Lab, Woods Hole, MA

Large plasma membrane disruptions (PMDs) rapidly invoke a localized exocytotic reaction that adds a 'patch' of internal membrane to the plasma membrane at the PMD site, a calcium-dependent resealing mechanism. We have used sea urchin eggs as a model system to define the mechanistic basis of this fundamental cell survival response. Here we directly observed plasma membrane tears that occur in sea urchin eggs during centrifugation with a special centrifuge polarizing microscope (CPM). Dilute suspensions of unfertilized eggs were layered in a centrifuge chamber above an osmotically matched dense solution containing Percoll, forming a density gradient that allowed the eggs to slowly settle to an equilibrium position. Centrifugation at speeds of up to 8,000 rpm for 20 min, separated the eggs into two parts. One part was filled with yolk granules and internal vesicles, the second part was filled with clear cytoplasm. These membrane tears by shear forces did not show variously shaped surface projections involved in exocytosis at the PMDs. These cell separations depended on the presence of calcium. However, sea urchin eggs were broken by this centrifugation in the absence of calcium. The part filled with yolk granules and internal vesicles repaired the PMDs made by a two-photon laser, but the part of the eggs containing clear cytoplasm did not repair. We conclude that shear forces caused by centrifugation make small PMDs which can reseal in the presence of extracellular calcium, although large PMDs made by laser irradiation require a 'patch' of internal membrane by calcium-dependent exocytosis.

P41**Board Number: B140****Harness human iPS cell differentiation and reprogramming potential using an automated cell culture system.**T. Guo¹, M. Watson¹, N. Devaraju¹, L. Szpankowski¹, B. Fowler¹, M. Norris¹, M. Thu¹, M. Wong¹, D. Kemp¹, H. Choi¹, B. Clerkson¹, G. Harris¹, Y. Lu¹, C. Johnson¹, A. Leyrat¹, X. Wang¹, G. Sun¹, M. Unger¹, R.C. Jones², N. Li¹;¹Fluidigm Corp, South San Francisco, CA, ²Fluidigm Corporation, South San Francisco, CA

In vitro studies using pluripotent stem cells provide an essential tool for understanding hard-to-study in vivo developmental processes. Human iPSCs also holds great potential for disease modeling and cell-based therapies. A major challenge in the stem cell field, however, is to define the optimal condition for cell expansion, differentiation, and reprogramming. Since multiple intra- and extra-cellular signaling

pathways are involved in each cellular process, a combinatorial approach to screen multiple factors is highly desirable. To facilitate the exploratory processes, we have developed an automated cell culture system for cell manipulation and environmental control. The system consists of an integrated fluidic circuit (IFC), an electro-pneumatic controller instrument, experimental designer software, and automated run-time control software. Each IFC has 32 culture microchambers and 16 reagent inlets. Each microchamber can be dosed separately with different combinations and ratios of the 16 reagents at various predefined time points. Using this system, we have developed a novel nonintegrating method for direct conversion of human BJ fibroblasts to neurons at high efficiency and cell viability using microRNA mimics (miR-9, 9*, 124) and synthetic mRNAs (NeuroD2, Ascl1, and Myt1L). The system is compatible with multiple extracellular matrices including Matrigel[®], vitronectin, and fibronectin, It allows 3-day hands-off operation and long term cell culture (more than three weeks). We demonstrated that human iPSCs can differentiate to neural progenitor cells or nociceptor neurons on the IFC after treatment with small molecules. Furthermore, using combinations of small molecules and signaling proteins in chemically defined media, we demonstrated directing human iPSCs into primitive lineages of all three germ layers on one IFC within four days. Immunostaining of marker proteins and single-cell gene expression analysis were used to profile the cell types. The results were consistent with published reports and confirmed in large well-dish format. Finally, we have also developed protocol for RNA transfection that allows efficient delivery of exogenous mRNA such as nGFP into human iPSCs as well as successful siRNA knockdowns of either endogenous or exogenous genes. Using a siRNA against the pluripotent gene OCT4, we repeatedly demonstrated more than 50% knock-down in both RNA and protein levels of the gene, providing an easy approach for gene manipulation within human iPSCs. In summary, the automated microfluidic platform employs precise control of microenvironment of cells, facilitates studies of multifactorial combinations, and enables development of robust, reproducible, and chemically defined cell culture and manipulation.

P42

Board Number: B141

Optical clearing of mouse skeletal muscle with the CLARITY method.

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Skeletal muscle has several levels of organization that may be specifically modified during development or in diseases, from the myofibrils to the whole muscle. It may be interesting, for example, to visualize the pattern of innervation or vascularization of a whole muscle. Fluorescent proteins conjugated to muscle proteins can be expressed in skeletal muscles and can be visualized in single dissociated fibers, in small bundles of fibers, or in vivo within a millimeter or less of the muscle surface. However, they cannot be localized in whole muscles, which are opaque to light. We now report that we have successfully applied the CLARITY method (Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging/Immunostaining/In situ hybridization-compatible Tissue-hydrogel; Chung et al., 2013, Nature 497, 332) to the clearing of the soleus and extensor digitorum longus (EDL) muscles of the mouse. The

CLARITY method we applied toward muscle tissue clarification is an adaptation of a technique originally used to clarify soft, lipid-rich brain tissue. In our application, the muscles were treated as described in the original technique, with small modifications: incubation in a solution containing a hydrogel monomer, paraformaldehyde, and a thermal initiator for 3 days at 4°C; followed by gelling at 37°C and clearing by electrophoresis at 23V for 8h. So far, attempts to label actin myofilaments in the whole muscle with fluorescent phalloidin, as well as neuromuscular junctions with Alexa-555-conjugated bungarotoxin, have failed. However, nuclei could be viewed after staining with Hoechst 33242 and myosin heavy chain bands could be imaged in 2-photon Second Harmonic Generation (SHG) imaging, albeit with reduced intensity compared to intact muscle. Thus it is possible to clarify a tissue like skeletal muscle, rich in connective tissue and filamentous proteins and low in fat. Furthermore basic muscle fiber organization and the myosin heavy chain organization responsible for SHG are maintained in the clarified muscle.

P43

Board Number: B142

Monitoring perturbations in endoplasmic reticulum calcium homeostasis using a novel secreted reporter protein.

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Calcium in the endoplasmic reticulum (ER) is maintained at a concentration approximately 10,000-fold greater than the cytoplasm. ER calcium homeostasis is critical for cellular functions and is disrupted in diverse pathologies including diabetes, cardiovascular diseases, and neurodegenerative diseases. Defining the relationship between disease progression and ER calcium imbalance, however, is challenging due to technological limitations. We have developed a tool that allows the examination of ER calcium fluctuations over extended times, which we have termed SERCaMPs, or secreted endoplasmic reticulum calcium monitoring proteins. Our studies identified a carboxy-terminal amino acid sequence (ASARTDL) that localizes a secreted protein to the ER and confers release of the protein specifically in response to ER calcium depletion. This sequence was appended to Gaussia luciferase (GLuc), a naturally secreted luciferase protein, to create GLuc-SERCaMP. Monitoring the secretion of GLuc-SERCaMP provides a simple method to assess ER calcium homeostasis *in vitro* or *in vivo* by collecting and measuring extracellular fluids such as culture medium or blood. GLuc-SERCaMP signal is robust, with detection of ER calcium depletion possible in as few as twenty cells in culture. We assessed the utility of GLuc-SERCaMPs in models of ER stress, including hyperthermia and glutamate toxicity, in rat primary neurons. Additionally, ER calcium homeostasis in rat liver was examined by measuring release of the reporter into blood. Our results suggest that SERCaMPs will have broad applications for the long term monitoring of ER calcium homeostasis and the development of therapeutic approaches to counteract ER calcium dysregulation.

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Actin and Actin-Associated Proteins 1

P44

Board Number: B144

An Alternative Mechanism for Actin Arc Formation at the Immunological Synapse of Jurkat T cells.

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Actin cytoskeletal organization and dynamics play fundamental roles in T cell immunological synapse (IS) formation. The T cell's actin cytoskeleton undergoes striking rearrangements upon contact with an antigen presenting cell (APC) to form radially symmetric segregated domains named the distal, peripheral and central supramolecular activation clusters (dSMAC, pSMAC, and cSMAC). The outer dSMAC corresponds to a lamellipodial (LP) actin network composed of branched actin arrays that undergo robust, polymerization-driven actin retrograde flow. The central pSMAC corresponds to a lamellar (LM) actin network composed of concentric actin arcs that undergo actomyosin II-driven contraction. Finally, the inner cSMAC is largely devoid of actin. This bull's-eye pattern of SMACs generates a centripetal flow of actin that drives the inward movement of signaling and adhesion molecules to create a mature IS. Here we addressed how the concentric, linear actomyosin II arcs in the pSMAC are assembled. Current thinking would have it that the branched actin network in the dSMAC, which is created by Arp2/3-dependent nucleation, is converted by debranching and crosslinking into the concentric arcs. Using structured illumination microscopy, we identified for the first time linear actin filaments that are arranged perpendicular to the plasma membrane and that are embedded in the branched actin network in the dSMAC. As these filaments exit the inner aspect of the dSMAC, they splay out and reorient into concentric arcs with an inherent antiparallel organization required for myosin II-dependent contraction. The perpendicular actin filaments in the dSMAC are highlighted and accentuated by inhibiting Arp2/3-dependent actin polymerization using CK666, which collapses the branched actin array in between the perpendicular filaments. Importantly, inhibition of myosin II using blebbistatin results in loose, disorganized filaments in the pSMAC that fail to reorient into concentric arcs. These observations suggest that arc assembly can occur independently of Arp2/3-dependent nucleation, and that myosin II contractility is required for reorienting the perpendicular filaments emanating from the dSMAC into the concentric arcs in the pSMAC. We are now attempting to define the relative contributions of debranching/rearrangement of the branched actin network versus nucleation of linear filaments, possibly by the formin INF2 present on the plasma membrane at the tips of the perpendicular filaments, to the formation of the actin arcs in the pSMAC.

P45**Board Number: B145****A Coarse-Grained Model of Lamellipodium Protrusion and Retraction Driven by Fluctuations in Actin Polymerization.**G.L. Ryan¹, D. Vavylonis², N. Watanabe³;¹Department of Physics, Kettering University, Flint, MI, ²Department of Physics, Lehigh University, Bethlehem, PA, ³Tohoku Univ Grad Sch Life Scis, Sendai, Japan

Animal cells that spread onto a surface often rely on actin-rich cell extensions called lamellipodia to execute cell protrusion. XTC cells on a two-dimensional substrate exhibit regular protrusion and retraction of their lamellipodium, even though the cell is not translating. Travelling waves of protrusion have also been observed, similar to those observed in crawling cells. These periodic fluctuations in leading edge position have been linked to excitable actin dynamics near the cell edge using a one dimensional model of actin dynamics, as a function of arc-length along the cell. In this work we extend this earlier model of actin dynamics into two-dimensions (along the arc-length and radial directions of the cell) and include a model membrane that protrudes and retracts in response to the changing number of free barbed ends of actin filaments near the membrane. We show that if the polymerization rate of these barbed ends changes depending on their local concentration at the leading edge and the opposing force from the cell membrane, the model can reproduce the patterns of membrane protrusion and retraction seen in experiment. We investigate both Brownian ratchet and switch-like force-velocity relationships between the membrane load forces and actin polymerization rate.

P46**Board Number: B146****The role of filopodia in directed cancer cell migration in 2D and 3D.**A. Simon¹, S. Geraldo², P. Maiuri², a. funfak³, j. bibette³, m. piel¹, D. Vignjevic²;¹Inst Curie-Ctr De Recherche, Paris, France, ²Institut Curie, Paris, France, ³75005, ESPCI, PARIS, France

When cancer cells escape the primary tumor, they invade into the surrounding tissue and migrate through the extracellular matrix to reach the circulatory system. It is, therefore, conceivable that cancer cells migrate directionally towards the blood vessels. Directional guidance of cells following gradients of growth factors and chemokines are known to exist in vivo during embryonic development and immune response. Filopodia are thought to be the guidance organelles for directional migration through gradients. However, besides studies in neuronal and endothelial cells, there is no evidence confirming this hypothesis in other cell types, such as cancer cells. Using stable CT26 intestinal cancer cell line as a model for invasive tumorigenesis, we investigated if filopodia have a role in directed cancer cell migration in 2D and 3D substrates. The expression of proteins involved in filopodia formation and/or stabilization was inhibited in order to study the ability of depleted cells to move towards a source of growth factors. The different depleted cell lines were compared for their ability to perform chemotactic movements on a planar substrate towards a source of growth factors, using the Dunn's chamber. WT

cells were able to move directionally and persistently towards the attractants, whereas cells depleted for Fascin, mDia2 and MyosinX were not able to migrate directionally towards the gradient. These results show that filopodia are necessary for directional migration of cancer cells on 2D. To assess the role of filopodia in invasive migration of cancer cells through 3D environments, we embedded cancer cell spheroids in 3D matrices of collagen I. After 48 hours, WT cells present typical mesenchymal morphology, invading and migrating between the collagen fibers in a stellar pattern radiating from the spheroid periphery. Only Eps8 and MyoX depleted cells presented a round morphology in 3D and a dramatic decreased invasion of collagen 3D matrices. Finally, we are currently assessing if filopodia are guidance organelles for cancer cells migration in 3D matrices. For this study we are using a microfluidic 3D chemoinvasion chamber allowing the formation of long-term gradients of growth factors in a collagen matrix.

P47

Board Number: B147

Retinomotor movements and the distribution of the visual enzyme, RPE65, in fish retinal pigment epithelial cells.

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Eyes of lower vertebrates, including fish, lack a dilatable pupil to regulate light flux. Instead, dramatic retinomotor movements occur, which involve movement of melanin pigment granules in the retinal pigment epithelium (RPE) and elongation and contraction of photoreceptor inner and outer segments. When exposed to light, pigment granules create a moveable screen and disperse throughout the RPE elongated apical projections. Rods, responsible for dim light vision, elongate and are enveloped by pigment-filled RPE. Cones, which are responsible for color and bright- light vision, contract, putting them first in line to light entering the eye. In the dark, these retinomotor movements are reversed. RPE pigment granule migration and the positional rearrangements of photoreceptors are actin- and microtubule-dependent. A different type of light-dependent motility in RPE of mammalian eyes was reported in a recent study. RPE contains an isomerase, RPE65, which regenerates the all-trans retinal chromophore back to its cis-form, crucial for the visual cycle. RPE65 localizes towards the apical side of mouse RPE in the dark and redistributes to the basal side in the light. This motility appears to be an actin-myosin dependent process, requiring the motor protein, myosin VIIa (Lopes, et al. 2011, Hum Mol Gen, 20: 2560). Since pigment granule movement in fish RPE also requires actin and is much more extensive than in mammalian eyes, we are investigating whether RPE65 similarly undergoes light-dependent translocation in fish RPE. In dissociated, isolated fish RPE cells *in vitro*, RPE65 was identified in apical projections using immunofluorescence, however no change in RPE65 distribution was observed in cells triggered to aggregate or disperse pigment granules. To determine if RPE65 undergoes redistribution in light- and dark- adapted eyes *in situ*, we labeled cryosections of RPE plus retina. Although RPE65 labeling in the RPE was visible, melanin pigment quenched some fluorescence. We are

currently working with albino catfish RPE-retina to eliminate issues with pigment quenching and to characterize RPE65 distribution in fish RPE.

P48

Board Number: B148

TRIM9 Regulates Filopodia During Cortical and Retinal Development.

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Early in neuronal development actin-rich filopodial protrusions are a critical prerequisite for neurite formation. Later, as nascent axons extend toward their postsynaptic partners, filopodia are central in growth cone response to extracellular axon guidance cues such as netrin-1. The netrin-1 receptor DCC localizes to axonal filopodia tips and mediates netrin-1 dependent filopodia formation and elongation as well as subsequent growth cone chemoattraction toward higher concentrations of netrin-1. We previously found that the E3 ubiquitin ligase TRIM9 binds DCC and is an important regulator in netrin-1 attractive signaling in murine cortical neurons. We show that TRIM9 interacts with actin regulatory proteins that promote filopodia formation, including members of the Ena/VASP family and Myo10, and that TRIM9 regulates filopodia number. Whereas the growth cones of cortical neurons typically have low basal numbers of filopodia that increase in response to netrin-1 stimulation, cortical neurons isolated from *TRIM9* knockout mice have elevated growth cone filopodia number that fail to respond to netrin-1. As such, *TRIM9*^{-/-} neurons morphologically resemble wild-type neurons treated with netrin-1 but lack sensitivity to netrin-1. Using high resolution time-lapse microscopy, we analyzed filopodia dynamics in cortical neurons; this revealed that loss of *TRIM9* increases the lifetime of individual filopodia, as does treatment of wild-type neurons with netrin-1. To determine if TRIM9-mediated regulation of filopodia lifetime and response to netrin-1 were conserved in other neuronal types, we investigated the role of *TRIM9* in retinal development. Retinal ganglion cells (RGCs) are another neuron type for which netrin-1 signaling is critical for axon guidance into the optic nerve. Cultured RGCs from *TRIM9*^{-/-} embryos again exhibited increased filopodia length compared to controls and failed to appropriately respond to netrin-1. Genetic loss of *TRIM9* results in a thinning of the RGC nuclear and nerve fiber layers in the adult murine retina as well as reduction in the number of RGC nuclei *in vivo*. Our findings suggest that TRIM9 is critical in the development of many neuronal types as well as in regulating filopodial responses to netrin-1 signaling. Current experiments are aimed to determine the mechanisms of TRIM9 mediation of pathways in netrin-1 signaling, as well as its relation to other neuronal guidance cues involved in retinal development such as Slits.

P49**Board Number: B149****Actin filaments are necessary for maintaining the normal structure of apical tubulobulbar complexes in the seminiferous epithelium.**K. Lyon¹, E. Bosseboeuf², A. Vogl¹;¹Cellular and Physiological Sciences, UBC, Vancouver, BC, ²Sciences Fondamentales et Appliquées, University de Poitiers, Poitiers, France

Here we explore the effects of actin perturbation on the morphology of tubulobulbar complexes in apical regions of the seminiferous epithelium. Tubulobulbar complexes are elongate subcellular machines responsible for the internalization of intercellular junctions during sperm release. Each tubulobulbar complex consists of a double membrane tubular core terminating in a clathrin coated pit. The membrane core is surrounded by a network of actin filaments and a distinct swelling or bulb develops in the distal third of the structure. Isolated testes were perfused for one hour with oxygenated Krebs Henseleit buffer (with BSA) at 33°C containing either cytochalasin D (10, 20 or 40 uM) or DMSO (control). They were then perfusion fixed either for fluorescence or electron microscopy. Quantification of fluorescently labeled actin at apical tubulobulbar complexes was done using thresholding techniques and demonstrated that the structures at stage VII of spermatogenesis were associated with lower levels of actin in experimental spermatids compared to controls. When evaluated at the electron microscopic level, the actin networks appeared patchy and expanded further away from the membrane cores than in controls. Ectopic bulb regions were also seen near the bases of the complexes in experimentally treated tissue. We conclude that the actin networks are necessary for maintaining the normal structure of apical tubulobulbar complexes. Supported by a NSERC Discovery Grant to AWV.

P50**Board Number: B150****Mutations in *Drosophila* crinkled/Myosin VIIA disrupt denticle morphogenesis.**J.L. Sallee¹, J. Crawford², V. Singh², D.P. Kiehart³;¹North Central College, Naperville, IL, ²Biology, Duke University, Durham, NC, ³Duke University, Durham, NC

Actin filament crosslinking, bundling and motor proteins are necessary for the assembly of epithelial projections such as microvilli, stereocilia, hairs, and bristles. Mutations in such proteins cause defects in the shape, structure, and function of these actin-based protrusions. One protein, Myosin VIIA, is an actin-based motor protein conserved throughout phylogeny. In *Drosophila melanogaster*, severe mutations in crinkled (ck) are “semi-lethal” with only a very small percentage of flies surviving to adulthood. Such survivors show morphological defects related to actin bundling in hairs and bristles and are deaf. To better understand ck/MyoVIIA’s function in bundled-actin structures, we used dominant female sterile approaches to analyze the loss of maternal and zygotic ck/MyoVIIA in the morphogenesis of denticles, small actin-based projections on the ventral epidermis of *Drosophila* embryos.

Maternal/zygotic ck/MyoVIIA mutants displayed severe defects in denticle morphology – actin filaments initiated in the correct location, but failed to elongate and bundle to form normal projections. Using deletion mutant constructs, we demonstrated that the motor domain and both of the C-terminal MyTH4 and FERM domains are necessary for proper denticle formation. Furthermore, we showed that ck/MyoVIIA interacts genetically with dusky-like (dyl), a member of the ZPD family of proteins that links the extracellular matrix to the plasma membrane. Loss of one protein does not alter the localization of the other; however, loss of the two proteins together dramatically perturbs denticle shape. Our data indicate that ck/MyoVIIA plays a key role in arranging actin filament bundles into biologically functional units, which drive proper shape of cellular projections. Supported by National Institutes of Health GM33830 and NIDCD007673 to DPK and F32GM093592 to JLS.

P51

Board Number: B151

Dystroglycan depletion inhibits the functions of differentiated HL-60 cells.

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Haematopoiesis involves the coordination of several signal transduction pathways, which are induced by extracellular stimuli through cell–cell and cell–extracellular matrix (ECM) interactions. Interactions with the ECM binding partners represent the critical steps by which cells initiate cytoplasmic signaling essential for the regulation of their growth, differentiation, attachment and migration, and are important factors in the development and progression of many types of cancer. Dystroglycan (Dg) is an adhesion molecule responsible for crucial interactions between ECM and the cytoplasmic compartment. It is formed by two subunits, α -Dg (extracellular) and β -Dg (transmembrane), that bind respectively to laminin in the matrix and to one of several cytolinker proteins such as dystrophin with the cytoskeleton. As an integral part of the dystrophin glycoprotein complex (DGC) of skeletal muscle, dystroglycan is essential for signaling events mediated through this complex that may have a role as a mechanochemical, force-transducing system or shock-absorber and indirectly mediating other signaling pathways associated with the DGC. Dystroglycan has recently been characterized in blood tissue cells, as part of the dystrophin glycoprotein complex but to date nothing is known of its role in the differentiation process of neutrophils. We have investigated the role of dystroglycan in the human promyelocytic leukemic cell line HL-60 differentiated to neutrophils. Depletion of dystroglycan by RNAi resulted in altered morphology and reduced properties of differentiated HL-60 cells, including chemotaxis, respiratory burst, phagocytic activities and expression of markers of differentiation. These findings strongly implicate dystroglycan as a key membrane adhesion protein involved in the differentiation process in HL-60 cells and so is therefore of considerable functional importance.

P52**Board Number: B152****Discoidin domain receptor 1 controls linear invadosome formation.**

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Accumulation of type I collagen fibrils in tumors is associated with an increased risk of metastasis. Invadosomes are F-actin structures able to degrade the extracellular matrix. We previously found that collagen I fibrils induced the formation of peculiar linear invadosomes in an unexpected integrin-independent manner, raising the question of the receptor involved. Here, we show that Discoidin Domain Receptor 1 (DDR1), a collagen receptor overexpressed in cancer, co-localizes with linear invadosomes in tumor cells and is required for their formation and matrix degradation ability. Unexpectedly, DDR1 kinase activity was not required for invadosome formation or activity, nor was Src tyrosine kinase. We show that the RhoGTPase Cdc42 localizes to linear invadosomes, is activated upon plating cells on collagen in a DDR1-dependent manner, and is required for linear invadosome formation. Finally, DDR1 depletion blocked cell invasion in a collagen gel. Altogether, our data uncover the important role of DDR1, acting through Cdc42, in proteolysis-based cell invasion in a collagen-rich environment.

P53**Board Number: B153****Metabolic filaments, the actin cytoskeleton, and orphan diseases.**

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A core question in disease research is to determine how the cell is altered on a molecular level in affected individuals. In the case of diseases caused by inborn errors of metabolism, this means identifying the relationship between activity of the affected enzyme and the observed disease phenotypes. Recently, we uncovered nearly 50 previously unidentified, conserved filamentous structures including eight novel filament systems that are comprised of enzymes with previously described roles in cellular metabolism. Because these enzymes are implicated in intermediate metabolism, we have focused on answering two key questions. First, have these filaments acquired any cell biological or cytoskeletal role that is separable from the known function of the enzyme. Second, are there any inborn errors of metabolism whose phenotypes can be explained by understanding the function of these polymers. To answer these questions, we have focused our efforts on PRPP synthase, a metabolic enzyme that forms filaments that are conserved from yeast to humans. Interestingly,

Human PRPP synthase forms a novel nuclear filament. Since there are no known roles for PRPP in the nucleus, this suggested the filament might have a specific nuclear function. Furthermore, there are several classes of human disease mutations in PRPP synthase - overexpression mutations which cause gout, loss of function mutations that cause deafness and ataxia, and feedback resistance mutations that cause gout, deafness, and ataxia. The fact that feedback resistance mutations cause both gain and loss of function phenotypes also suggested that PRPP synthase filaments might have acquired a second function separable from its enzyme activity. Using fibroblasts from human patients, we have found that superactivity mutations cause PRPP synthase to polymerize in the cytoplasm where filaments are not normally found. These filaments are not nonfunctional aggregates since treatment with purine metabolites can cause reversible disassembly of the filaments. This suggests that superactivity mutations can generate dominant negative filaments in the cytoplasm leading to a loss of function phenotype. Additionally, we have identified a protein that co-localizes with PRPP synthase filaments that also appears to be required for *Drosophila* bristle formation, suggesting a link between purine metabolism and actin cytoskeleton regulation. This provides the first molecular explanation for how mutations in a purine biosynthetic enzyme can cause non-syndromic deafness in humans and opens the door for exploring how other metabolic polymers might explain the symptoms associated with many inborn errors of metabolism.

P54

Board Number: B154

Actin Cytoskeletal Dynamics Regulate Adhesion and Force Generation in Airway Smooth Muscle Cells.

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Actin dynamics in non-muscle cells have been well characterized and shown to play crucial roles in regulation of cell adhesion, migration and force generation. Despite this extensive knowledge, much less is known about the regulation of the actin cytoskeleton in smooth muscle cells. Adhesion and force generation by smooth muscle cells are crucial to many physiological functions and contribute to the pathology of numerous diseases, including asthma. Here we demonstrate that adherent human airway smooth muscle cells (hASM) have an actin cytoskeleton that is highly reminiscent to that of non-muscle cells. The cell periphery is dominated by an Arp 2/3-mediated lamellipodium while the cell center is characterized by a myosin-rich and formin-mediated lamella. Moreover, these actin-based organelles control cell adhesion and force generation in a similar fashion to their role in non-muscle cells. Arp2/3 inhibition eliminates the lamellipodium and results in abrogated nascent adhesion assembly and a reduction in hASM cell adhesion. Inhibition of formin-mediated actin assembly results in a decrease in lamellar actin by at least 25 percent. This formin-dependent decrease in lamellar actin results in a 60

percent decrease in contractility of hASM cells. This work suggests that models of non-muscle cell contractility may be useful in understanding the mechanics of airway smooth muscle cells.

P55

Board Number: B155

Distinct Actin Filament Networks Modulate Different Cell Signalling Pathways Involved in Cell Proliferation and Senescence.

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The spatial and temporal control of signalling pathways that regulate cellular processes, including proliferation and senescence, is orchestrated by a complex network of interactions involving scaffolding proteins and kinases. The actin cytoskeleton is known to play a critical role in integrating such signalling pathways. Within a cell functionally diverse actin filament networks are present. Currently, the molecular mechanism(s) by which these actin networks participate in signalling pathways is not well-understood principally due to a lack of tools that discriminate between them. Tropomyosins (Tm), a family of actin filament-associated proteins, can impart unique cellular functions to the actin cytoskeleton. Our investigations using mouse embryonic fibroblasts (MEFs) isolated from Tm isoform-specific knockout (KO) and transgenic mice have revealed significant alterations in cell proliferation and life span. Ablation of Tm5NM1 results in reduced cell proliferation in direct contrast to its overexpression. This result is Tm5NM1-specific since ablation of Tm4 shows no impact on proliferation. We show that Tm5NM1-mediated proliferation occurs via the MEK/ERK pathway. Furthermore we demonstrate that in the absence of Tm5NM1 the formation of pERK and its nuclear shuttling protein Importin 7 is reduced resulting in the observed impaired cell proliferation. In contrast, an in vitro life span assay demonstrates that Tm4 KO MEFs can readily escape senescence unlike the Tm5NM1 KO cells. The Tm4 KO MEFs show dysregulation of the PKC and JAK-STAT pathways. These studies provide the first evidence that specific Tm-containing actin filaments can influence signalling pathways. Taken together these findings allow us to begin to understand how signalling pathways are regulated within the cell in space and time via different actin filament networks marked by specific Tm isoforms.

P56**Board Number: B156****Zyxin mediated regulation of airway smooth muscle function may be involved in asthmatic airway hypercontractility.**

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Morbidity and mortality due to asthma arise mainly from loss of airway patency as a result of airway hypercontractility. Airway hypercontractility is the result of excessive contraction of airway smooth muscle. Airway hypercontractility in the non-asthmatic lung is readily reversed by the taking of a deep breath, which results in bronchodilation. A deep breath stretches the contracted airway smooth muscle causing it to fluidize rapidly and then resolidify slowly. On the other hand, airway hypercontractility in the asthmatic lung is generally refractory to the dilative effect of a deep breath. In the asthmatic case, the airway smooth muscle enters what is called a latch state, wherein the parenchymal tissue lacks sufficient tethering force to stretch the airway smooth muscle. The molecular mechanisms of stretch induced airway smooth muscle fluidization and resolidification are unknown, as is the mechanism of its failure in asthmatics.

The LIM-domain scaffolding protein zyxin has been shown to be involved in repairing, remodeling and reinforcing actin stress fibers in response to mechanical strain. Thus, zyxin is essential for generation of peak traction forces. Zyxin is rapidly recruited to sites of stress fiber strain, where it enlists actin regulatory proteins to effect repair. Due to its role in regulation of the actin cytoskeleton's response to force, we hypothesized zyxin might be involved in the regulation of the airway smooth muscle response to stretch. In this work we show that zyxin plays a critical role in reinforcing the actin cytoskeleton of airway smooth muscle in response to stretch. We demonstrate that zyxin responds to physiologic levels of isotropic stretch through rapid recruitment to sites of acute strain on stress fibers, and in murine airway smooth muscle, loss of zyxin results in abrogated resolidification following single or multiple stretches. In precision cut lung slices from wild type or zyxin null mice, the airways from zyxin null mice dilate more rapidly in response to stretch. Furthermore, zyxin expression is increased in the lungs of humans who experienced fatal asthma attacks.

Together, these data show a role for zyxin in the regulation of airway smooth muscle response to stretch, and suggest a connection to airway hypercontractility in human asthmatics.

P57**Board Number: B157****Synthetic polyamines promote rapid lamellipodial growth by regulating actin dynamics.**D. Riveline¹;¹Laboratory of Cell Physics ISIS/IGBMC, Strasbourg, France

Cellular protrusions involved in motile processes are driven by site-directed assembly of actin filaments in response to Rho-GTPase signalling. So far, only chemical compounds depolymerizing actin or stabilizing filaments, inhibiting N-WASP, Arp2/3 or formins, have been used to eliminate the formation of protrusions, while Rho-GTPase-dominant positive strategies have been designed to stimulate protrusions. Here we describe the design of four polyamines (macrocyclic and branched acyclic), and show that they enter the cell and induce specific growth of actin-enriched lamellipodia within minutes. The largest increase in cell area is obtained with micromolar amounts of a branched polyamine harbouring an 8-carbon chain. These polyamines specifically target actin both in vitro and in vivo. Analysis of their effects on filament assembly dynamics and its regulation indicates that the polyamines act by slowing down filament dynamics and by enhancing actin nucleation. These compounds provide new opportunities to study the actin cytoskeleton in motile and morphogenetic processes.

- Nedeva, I., G. Koripelly, D. Caballero, L. Chieze, B. Guichard, B. Romain, E. Pencreach, J.M. Lehn, M.F. Carrier, and D. Riveline. 2013. Synthetic polyamines promote rapid lamellipodial growth by regulating actin dynamics. Nat Commun. 4:2165.

P58**Board Number: B158****Biomechanical characterization of a new cellular model for Filamin C-related myofibrillar myopathy.**W.H. Goldmann¹, F. Chevessier-Tuennesen², L. Einsiedler^{1,2}, R. Schröder², B. Fabry¹;¹Friedrich-Alexander-Univ/Ctr Med Physics Technol, Erlangen, Germany, ²Institute of Neuropathology, University Hospital Erlangen, Erlangen, Germany

Mutations on chromosome 7q32 of the human filamin C gene (FLNC) are responsible for an autosomal dominant form of myofibrillar myopathy. The expression of mutant W2710X filamin C, the most common pathogenic mutation in humans, leads to the disruption of Z-discs of striated muscle sarcomeres and abnormal protein aggregates. We tested whether the expression of mutant filamin C protein changes the biomechanical properties and the intrinsic mechanical stress response of immortalized myogenic cells derived from heterozygous and homozygous filamin C knock-in mice bearing the human homologous W2711X mutation. Compared to wildtype cells, mutant filamin C myoblasts and myotubes showed increased cell death and substrate detachment in response to cyclic stretch on flexible membranes. Moreover, 2D force microscopy of murine myoblasts showed lower

tractions in mutated compared to wildtype cells. Our findings provide first evidence that altered mechanical properties may contribute to the progressive striated muscle pathology in filaminopathies. We postulate that the expression of mutant filamin C leads to altered biophysical properties making these mutated myogenic cells more vulnerable to mechanical stress.

P59

Board Number: B159

Determining the impact of the actin cytoskeleton on the mechanical properties of cells.

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The influence of the actin cytoskeleton on the shape and mechanical properties of cells has been revealed extensively using anti-actin drugs. However, due to the lack of the specificity of these compounds for different actin filament populations it has been difficult to delineate the exact role of the actin cytoskeleton in these cellular features. Actin binding proteins largely affect the organisation and dynamics of actin in cells; however, the impact of these proteins on the mechanical properties of cells is still unknown. Tropomyosins (Tms) form a co-polymer with actin filaments and differentially regulate actin filament stability, function and organisation through their highly regulated expression and sorting to specific intracellular sites. Hence, Tm isoforms can be used as tools to identify and manipulate functionally distinct actin filament populations in a cell. In this study we investigated the impact of Tm-containing actin filaments on the organisation of the actin cytoskeleton and the mechanical properties of cells.

To investigate the role of different Tms, and hence different actin filaments, on cellular stiffness we examined rat neuroblastoma cells stably overexpressing TmBr3, Tm3, Tm4 or Tm5NM1 in comparison with control cells. Analysis of the number and length of actin cables per cell using a morphometric linear feature detection algorithm revealed significant differences in the length and number of actin cables per cell relative to that seen in the control cells. The mechanical measurements conducted by indentation-type atomic force microscopy (AFM) showed that TmBr3-, Tm4- and Tm5NM1-overexpressing cells were significantly stiffer than control cells. In contrast, cell stiffness did not change in Tm3-overexpressing cells indicating that the observed changes in cell elasticity are not a generic effect of accumulating more Tm. Moreover, siRNA knockdown of Tm5NM1 in Tm5NM1-overexpressing cells caused the elastic modulus to revert to that of control cells.

The results show that cell stiffness increases in TmBr3-, Tm4- and Tm5NM1-overexpressing cells. Since, the overexpression of Tm changes the numbers and length of the actin cables in an isoform-specific manner that is not directly related to cell stiffness, our data suggest that the mechanical properties of cells are more dependent on the organisation of the actin filaments than the number and length of actin

filaments. Therefore, we conclude that it is specific aspects of actin organisation which regulate the mechanical properties of the cell.

P60

Board Number: B160

Dynamic actin filaments control the mechanical behavior of the human red blood cell membrane.

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The mammalian red blood cell (RBC) contains an underlying membrane skeleton that imparts the RBC with mechanical properties optimized for efficient passage through the microvasculature. The RBC membrane skeleton consists of nodes of short (~37-nm-long) actin filaments interconnected by long ($\alpha_1\beta_1$)₂-spectrin tetramer strands. RBC actin filaments are stabilized along their lengths by two tropomyosin isoforms, TM5NM1 and TM5b, and capped at their pointed and barbed ends by tropomodulin1 and $\alpha\beta$ -adducin, respectively. However, it remains unknown whether RBC actin filaments (or subsets or portions thereof) are dynamic during normal RBC homeostasis, and, if so, whether actin dynamics regulates RBC mechanical properties. To answer these questions, we first studied actin dynamics by rhodamine-actin (rho-actin) incorporation into RBC ghosts. We also treated RBCs or ghosts with three different actin-disrupting drugs: (i) cytochalasin-D (CytoD), which inhibits actin subunit association and dissociation at barbed ends; (ii) latrunculin-A (LatA), which destabilizes and depolymerizes dynamic actin filaments by binding to and sequestering actin monomers, driving the F:G-actin balance toward the G-actin state; (iii) jasplakinolide (Jasp), which stabilizes dynamic actin filaments, driving the F:G-actin balance toward the F-actin state. Results show that resealed RBC ghosts exhibit rho-actin subunit incorporation into discrete sites within the membrane skeleton, demonstrating that RBC actin filaments are indeed dynamic. Moreover, rho-actin subunit incorporation can be blocked via treatment with CytoD, demonstrating that dynamic subunit exchange occurs at barbed ends. Consistent with dynamic filaments, treatment of intact RBCs with LatA or Jasp induces a ~2-fold increase or a ~60% decrease, respectively, in soluble actin levels. Using total internal reflection fluorescence (TIRF) microscopy, we observed diffuse F-actin staining in DMSO-treated (control) RBCs, whereas LatA (but neither CytoD nor Jasp) treatment induced dramatic rearrangement of the RBC actin network, as indicated by gaps in the F-actin staining pattern. This result suggests the presence of a dynamic subpopulation of RBC actin filaments. LatA treatment and selective disassembly of this subset of RBC actin filaments had no effect on RBC osmotic fragility, but both membrane deformability and spontaneous membrane oscillations (“flickering”) were increased. Collectively, these results provide the first direct evidence for the existence of a dynamic subpopulation of RBC actin filaments, and that actin filament architecture directly controls the mechanics of the human RBC membrane.

P61**Board Number: B161****Localization of IQGAP1 mutants to retracting versus protruding cell areas.**J. Schober¹, M. Reimer¹;¹Pharmaceutical Sciences, Southern Illinois University Edwardsville, Edwardsville, IL

IQGAP1 interacts with numerous binding partners through a calponin homology domain (CHD), a WW motif, IQ repeats, a Ras GAP-related domain (GRD), and a conserved C-terminal (CT) domain. Among various biological and cellular functions, IQGAP1 plays a role in cell-matrix interactions and actin cytoskeleton dynamics during membrane ruffling and lamellipodium protrusion. Phosphorylation in the CT domain regulates intramolecular interaction and IQGAP1 cellular activity. In a recent study, we discovered that IQGAP1 localizes to actively retracting edges, instead of protruding areas, in B16F10 mouse melanoma cells and some other cells types. In these current studies we examined localization of IQGAP1 mutants to retracting versus protruding areas in B16F10 cells. Cells were cotransfected with GFP-IQGAP1 Full Length (GFP-IQGAP1-FL), as an internal control, and one of five Myc-tagged IQGAP1 constructs (FL, S1441E/S1443D, Δ CHD, Δ GRD or Δ CT). The cotransfected cells were plated onto laminin for 30 minutes, stained with anti-Myc and anti-WAVE antibodies, and normalized fluorescence measurements were made in retracting and protruding areas. Retracting cell areas were defined as GFP-IQGAP1-FL positive and WAVE negative, while protruding cell areas were defined as GFP-IQGAP1-FL negative and WAVE positive. In retracting areas there were large decreases in both Δ GRD and Δ CT localization, a slight decrease in Δ CHD localization, and normal localization of the S1441E/S1443D mutant. In areas of cell protrusion there were large increases in both Δ GRD and Δ CT localization, and normal localization of Δ CHD and S1441E/S1443D mutants. These results indicate that two domains, GRD and CT, are essential for normal localization of IQGAP1 to retracting cell areas. Furthermore, our results are consistent with a model in which IQGAP1 in the areas of cell retraction is in the open, phosphorylated, conformation.

P62**Board Number: B162****Role of Rho/Rock/Myosin in Senescence Induced Migration.**I. Aifuwa¹, N. Longe¹, S. An², D. Wirtz^{3,4};¹Johns Hopkins University, Baltimore, MD, ²School of Public Health, Johns Hopkins University, Baltimore, MD, ³Physical Sciences-Oncology Center, Johns Hopkins University, Baltimore, MD, ⁴Department of Pathology and Oncology and Sydney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, MD

Cells induced into senescence exhibit a marked increase in the secretion of pro-inflammatory cytokines termed senescence-associated secretory proteins (SASP). Here we report that SASP from senescent stromal fibroblasts promote spontaneous morphological changes accompanied by an aggressive migratory behavior in originally non-motile human breast cancer cells. This phenotypic switch is

coordinated, in space and time, by a dramatic reorganization of the actin and microtubule filament networks, a discrete polarization of EB1 comets, and an unconventional front-to-back inversion of nucleus-MTOC polarity. SASP-induced morphological/migratory changes are critically dependent on microtubule integrity and dynamics, and are largely cooperated via the inhibition of cell contractility. RhoA/ROCK inhibition reduces focal adhesions and traction forces, but promotes a gliding mode of migration.

P63

Board Number: B163

Rapid Glucose Deprivation Immobilizes Active Myosin-V on Stabilized Actin Cables.

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Polarization of eukaryotic cells requires organelles and protein complexes to be transported to their proper destinations along the cytoskeleton. When nutrients are abundant, budding yeast grows rapidly transporting secretory vesicles for localized growth and actively segregating organelles. This is mediated by myosin-Vs transporting cargos along F-actin bundles known as actin cables. Actin cables are dynamic structures regulated by assembly, stabilization and disassembly. Polarized growth and actin filament dynamics consume energy. For most organisms, glucose is the preferred energy source and generally represses alternative carbon source usage. Thus upon abrupt glucose depletion, yeast shuts down pathways consuming large amounts of energy, including the vacuolar-ATPase, translation and phosphoinositide metabolism. Here we show that glucose withdrawal rapidly depletes ATP levels and the yeast myosin V, Myo2, responds by relocalizing to actin cables within 1 min, making it the fastest response documented. Myo2 immobilized on cables releases its secretory cargo, defining a new rigor-like state of a myosin-V in vivo. Only actively transporting Myo2 can be converted to the rigor-like state. Additionally, glucose depletion has differential effects on the actin cytoskeleton resulting in disassembly of actin patches with concomitant inhibition of endocytosis, and strong stabilization of actin cables, thereby revealing a selective and previously unappreciated ATP requirement for actin cable disassembly. A similar response is seen in HeLa cells to ATP depletion. These findings reveal a new fast-acting energy conservation strategy halting growth by immobilizing myosin-V in a newly described state on selectively stabilized actin cables.

P64**Board Number: B164****Tracking old and newly synthesized β -actin protein in three dimensional MDCK cyst cultures.**P. Vedula¹, A.J. Rodriguez¹;¹Federated Department of Biology, Rutgers University, Newark, NJ

In vivo, most simple epithelia are organized as a monolayer of cells surrounding a central lumen (e.g. intestinal epithelium, kidney tubules, mammary acini, alveolar sacs). *In vitro*, epithelial cells cultured in a 3-dimensional extra-cellular matrix develop into cysts and provide a model system to study epithelial morphogenesis and lumen formation. Previous studies have shown that several polarity proteins (apical and baso-lateral) along with components of the protein sorting machinery play a role in lumen initiation in MDCK cysts grown in matrigel. However the role of mRNA localization on protein synthesis and hence lumen formation and/or maintenance has yet to be determined. In this study, we use MDCK cysts in matrigel to address the role of β -actin mRNA zipcode on its protein synthesis. Utilizing the tetracysteine reporter system and biarsenical dyes – FIAsh and ReAsH, we were able to track β -actin protein synthesis temporally in the 3D cultures. This method provides valuable insight into the dynamic nature of actin based structures in a more physiologically relevant 3 dimensional culture system, thus opening the doors to how mRNA zipcode dependent protein synthesis regulates epithelial structure and function *in vivo*. In fact, we observe that newly synthesized β -actin protein labeled by ReAsH is localized predominantly at the lateral cell-cell junctions while the older protein labeled by FIAsh is distributed at the apical and lateral interfaces. This provides direct evidence for the long standing hypothesis that localized translation of β -actin does indeed dictate its preferential incorporation into different compartments in the cell controlling 3D epithelial morphogenesis.

P65**Board Number: B165****Investigating EB1 interactions with the actin cytoskeleton.**E. Alberico¹, H. Goodson¹;¹University of Notre Dame, Notre Dame, IN

Many cellular processes require coordination between the actin and microtubule cytoskeletons. Cell division and migration are two very important processes that require this type of coordination. Proteins such as mDIA1 and IQGAP1 are known to regulate these interactions. Our lab has found that EB1, a well characterized microtubule binding protein, can also directly interact with actin *in vitro*. Through bioinformatics and mutagenesis we have found that the EB1-actin binding site on EB1 partially overlaps the well characterized EB1-MT binding interface. We are currently investigating the role EB1 has in interacting with the actin cytoskeleton through characterization of EB1 mutants *in vivo*. Understanding the EB1-actin interaction *in vivo* will enhance our knowledge of cytoskeletal coordination and its role in a variety of cellular processes.

P66**Board Number: B166****Microtubule end-binding protein EB1 directly regulates collaborative actin assembly by APC and formins.**R. Jaiswal¹, H. Kim², D. Breitsprecher², B.L. Goode¹;¹Department of Biology, Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, MA, ²Brandeis University, Waltham, MA

Tight coordination of the microtubule and actin cytoskeletons is essential for many biological processes, including directed cell migration, neuronal process formation, spindle rotation, and polarized cell adhesion. However, in few cases have the underlying mechanisms controlling microtubule-actin crosstalk been defined. Earlier studies identified EB1 as a binding partner of APC (adenomatous polyposis coli) and formins (Su et al., 1995; Wen et al., 2004), which prompted us here to investigate EB1's potential effects on APC-formin-mediated actin assembly. Using both TIRF microscopy and bulk actin assembly assays, we observed that purified full-length EB1 directly inhibits the actin nucleation activity of C-terminal fragments of APC, as well as collaborative actin assembly by APC and formins. EB1 specifically inhibited the nucleation of new actin filaments without affecting the elongation rate of existing filaments. Further, multi-wavelength single molecule TIRF experiments showed that EB1 inhibits actin nucleation by blocking the interactions of actin monomers with APC. Additionally, we show that APC directly links actin filaments to microtubule seeds, and that EB1 disrupts this linkage. Together, our results suggest that EB1 may coordinate microtubule plus end dynamics with APC-mediated actin assembly. We are now dissecting EB1-APC interactions to develop the tools required for testing this model in vivo.

References:

Su LK, Burrell M, Hill DE, Gyuris J, Brent R, Wiltshire R, Trent J, Vogelstein B, Kinzler KW. APC binds to the novel protein EB1. *Cancer Res.* 1995, 55, 2972-2977.

Wen Y, Eng CH, Schmoranzler J, Cabrera-Poch N, Morris EJ, Chen M, Wallar BJ, Alberts AS, Gundersen GG. EB1 and APC bind to mDia to stabilize microtubules downstream of Rho and promote cell migration. *Nat Cell Biol.* 2004, 6, 820-830.

P67**Board Number: B167****The formins FHOD-1 and INFT-1 are important for ovulation and localize to the somatic gonad of *C. elegans*.**A. Hegsted¹, D.W. Pruyne¹;¹Department of Cell and Developmental Biology, SUNY Upstate Medical University, Syracuse, NY

Formins are actin nucleators, and many promote actin filament elongation. To determine the physiological role of each formin in the genetic model system *Caenorhabditis elegans*, single deletions of its non-essential formins were obtained. These deletions produced apparently healthy worms, which suggested redundant formin functions. We generated double formin mutants and found that worms doubly-mutated for the formin genes *fhod-1* and *inft-1* had smaller brood sizes (number of progeny) and movement defects. This project characterizes the effect of *fhod-1;inft-1* double mutation on brood size and the localization of FHOD-1 and INFT-1 within the somatic gonad. The worm gonad is composed of two symmetrical arms. Germ cells are produced in the distal gonad and migrate proximally. Myoepithelial sheath cells surround the distal and proximal gonad. The proximal sheath cells have contractile actin networks that move the developing oocytes towards the spermatheca. The spermatheca, an actin rich organ, is the site of fertilization. The developing embryo resides in the uterus until it is expelled through the vulva. FHOD-1 localizes to the vulval and uterine muscles, and the proximal sheath cells. INFT-1 is expressed at the spermatheca. An ovulation defect seen in the double mutant worms is endomitotic oocytes in the gonad arm (emo). This phenotype can be the result of a contractility defect in the proximal sheath cells and/or the spermathecae. We examined ovulation in the double mutant worms and saw that the maturing oocyte follows one of three paths: normal ovulation, engulfment by the spermatheca that breaks the oocyte, or no ovulation. This abnormal ovulation could be the reason for the smaller brood size. We hypothesize that FHOD-1 and INFT-1 are important for organizing actin in the myoepithelial sheath cells and the spermatheca to allow proper ovulation.

P68**Board Number: B168****Defining the roles of the formin Diaphanous in organizing the contractile ring and midbody ring during cytokinesis.**Y. Ruella Palm¹, D. d'Allard^{1,2}, G.R. Hickson^{1,2};¹UHC Ste-Justine, Montreal, QC, ²Universite de Montreal, Montreal, QC

Animal cells divide by the intricate process of cytokinesis. Initially, a Rho GTPase and actomyosin-dependent contractile ring (CR) drives the constriction at the cell equator until a stable midbody ring (MR) forms and matures to guide the separation of the two cells. The CR and MR are highly organized structures controlled by interactions among a network of Rho-dependent proteins. One such protein is Diaphanous (Dia), a formin that nucleates unbranched actin filaments, localizes to the CR and is essential

for cytokinesis. Nevertheless, it is unclear how Dia contributes to the organization of the CR and, potentially, to the subsequent formation of the MR.

We have examined the requirement for Dia and actin during cytokinesis by time-lapse video microscopy of *Drosophila* S2 cells expressing markers tagged with fluorescent proteins (FPs). Acute administration of the inhibitor of actin polymerization, Latrunculin A (LatA), defined a period of 20 minutes from closure of the CR during which the nascent MR requires dynamic actin: before this time, furrows regressed; after this time the MR remained stable in LatA. Moreover, a functional Dia-FP localized to the CR and nascent MR but not to the mature MR and the timing of its disappearance coincided with the transition to a LatA-insensitive MR structure. Depletion of Dia by RNAi slowed furrow ingression, enhanced furrow oscillations and prevented furrow completion. Live imaging of the F-actin probe, LifeAct-GFP, in Dia depleted cells revealed a marked decrease in equatorial F-actin at the time of normal CR formation, although a cortical flow of F-actin from the poles to the equator was subsequently observed. The CR components Myosin-FP and the scaffold protein Anillin-FP were still recruited to the equator of Dia-depleted cells but formed aberrant punctate structures, demonstrating a role for Dia in maintaining the normally uniform organization of the CR. Our prior truncation analysis has uncovered multiple mechanisms contributing to the localization of Anillin to the equatorial cortex (Kechad, El Amine). An N-terminal region of Anillin that contains both F-actin and myosin binding domains and that mediates the proper localization of Anillin to the CR, was no longer recruited to the cortex in Dia-depleted cells or in cells treated with LatA. Since the latter treatment did not prevent the recruitment of Dia-FP, this suggests that Dia-dependent F-actin polymerization is specifically required for the recruitment and retention of Anillin at the CR. This is likely an important role for Dia that provides clues as to the organization of the CR. Furthermore, our data are consistent with a model in which the regulated removal of Dia from the F-actin-dependent CR promotes the transition to the Anillin-dependent MR.

P69

Board Number: B169

Potent inhibition of Diaphanous formins by actin oligomers produced by the Actin Crosslinking Domain toxin of *V.cholerae*.

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Actin Crosslinking Domain (ACD) is produced by pathogenic strains of *Vibrio cholerae*, *Vibrio vulnificus*, and *Aeromonas hydrophila*. Upon delivery to the cytoplasm of host cells via Type I or Type VI Secretion systems, ACD catalyzes the formation of an amide bond between K50 and E270 of actin monomers,

resulting in the formation of actin oligomers of varying sizes. According to the current view on the ACD pathogenesis, the ACD activity leads to a slow failure of the cytoskeleton due to the gradual accumulation of non-functional oligomers leading to depletion of both monomeric and filamentous actin pools. Instead, we propose that ACD employs a hitherto unprecedented toxicity mechanism by converting cytoplasmic actin into highly toxic oligomers that specifically target key steps of actin dynamics. Particularly, we demonstrate that the oligomers inhibit with sub-nanomolar affinity actin polymerization controlled by Diaphanous formins. TIRFM experiments reveal that the formin controlled elongation is blocked in a concentration dependent manner in the presence of nanomolar concentrations of the oligomers. Profilin plays a key role in the observed inhibition and mDia1 formin constructs with fewer proline-rich regions in their FH1 domains are inhibited by the oligomers less efficiently. Accordingly, we demonstrate that at least some vital functions of the cytoskeleton (e.g. integrity of epithelial barriers) are deeply impaired when only a small fraction of cytoplasmic actin is crosslinked into oligomers. The effects are reproduced by formin-, but not ARP2/3 complex-specific small molecule inhibitors. Intriguingly, the pool-down analysis of HeLa cell lysates using double-tagged actin demonstrates that the oligomers exhibit selectivity towards certain human formins, suggesting the possibility of creating specific ACD-based and/or ACD mechanism-inspired formin inhibitors. To conclude, we propose that delivery of only a few copies of ACD into the cytoplasm of the host cell enables this enzyme to produce actin oligomers that possess the unique combination of properties that is neither observed in G- nor F-actin. Specifically, a tandem organization of the ACD-crosslinked actin oligomers and their ability to interact with G-actin binding proteins (e.g. profilin) confers them an abnormally high affinity to tandem G-actin binding proteins (e.g. tandem proline-rich and WH2 domains of formins, VASP, and NPFs) and enables them to actively interfere with the specific nucleation and/or elongation activities of these proteins. Thereby, ACD initiates a toxicity cascade that employs actin oligomers as second messengers, whose novel, “gain-of-function” inhibitory properties potentially “poison” cellular functions orchestrated by formin-mediated actin polymerization.

P70

Board Number: B170

A role of RhoA activation in IGF-I-induced muscular hypertrophy model.

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Skeletal muscle loses the mass during the aging process. IGF-I, insulin like growth factor-I, stimulates several signaling pathways in the differentiation and hypertrophy of muscle cells, and prevents the skeletal muscle atrophy accompanying with aging. Small GTPase RhoA has been known to be essential for muscle development, but its precise role remains unknown. Here, we analyzed the activation and role of RhoA in an IGF-I-induced muscular hypertrophy model. To construct the IGF-I-induced muscle hypertrophy model, C2C12 cells were differentiated for 48 hours followed by further 48 hours in

differentiated media with or without IGF-I. With the addition of IGF-I, C2C12 cells showed prominent hypertrophic changes, and increased 1.3-fold in the maximum diameter of myotube by facilitating the fusion of myoblast. To determine the activation level of RhoA, we performed a pull-down assay for RhoA · GTP. After 24 hours of IGF-I treatment, RhoA activity increased 2.0-fold compared with 0 hour, and increased 62% relative to non-treatment cells. To confirm whether the activated RhoA contributes to the hypertrophy, we transfected RhoA dominant-negative mutant (RhoA-DN) into C2C12 cells to inhibit RhoA activation by IGF-I. The expression of RhoA-DN reduced the hypertrophy induced by IGF-I, and impaired the expression of differentiation marker (MHC and M-cadherin) and p38 MAP-kinase activation. These data suggests that IGF-I promotes the muscular hypertrophy via RhoA activation in this model. To identify the binding proteins of RhoA in IGF-I-induced hypertrophic signaling, we designed RhoA tagged with promiscuous biotin ligase BirA (Roux et al. 2012). This technique may provide novel insights into the role of RhoA in the signal networks during the muscular hypertrophy.

P71

Board Number: B171

PIP2 is required for the stability of cleavage furrow-associated proteins during cytokinesis.

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Meiotic division during *Drosophila melanogaster* spermatogenesis serves as an excellent model for studying mechanisms of cytokinesis. Our previous studies showed that phosphatidylinositol phosphates (PIPs) act as regulators of this process. To define the role of phosphatidylinositol 4,5-bisphosphate (PIP2) in meiotic cytokinesis, we studied cleavage furrow-associated proteins such as Anillin, Sep2 (a septin) and Sqh (myosin regulatory light chain) in PIP2-depleted testes. Live imaging of spermatocytes depleted of PIP2 by ectopic expression of the Salmonella PIP2 phosphatase SigD revealed that Anillin, Sep2, and Sqh initially localize to the cleavage furrow, yet lose their cortical localization during furrow regression, leading to formation of multinucleate cells. Reduction in the abundance of these proteins in the furrow prior to furrow regression, as well as oscillations of GFP-Anillin and Sqh-GFP along the cortex, indicated a perturbation of cleavage furrow integrity. To confirm that these phenotypes resulted from PIP2 depletion, we examined the *Drosophila* PIP 5-kinase Skt1, which synthesizes PIP2. YFP-Skt1 localized to cleavage furrows of dividing spermatocytes. Male germ cells homozygous mutant for *skt1* were multinucleate, consistent with a role for Skt1 in cytokinesis. Moreover, dividing *skt1* mutant spermatocytes, like those expressing SigD, exhibited reduced levels of Anillin and Pnut (a septin) in the cleavage furrow. Taken together, our data suggest that PIP2 is synthesized at the cleavage furrow, where it serves to maintain association of furrow proteins with plasma membrane for successful completion of cytokinesis.

P72**Board Number: B172****The small GTPase Rap1 promotes cell movement rather than stabilizes adhesion in epithelial cells responding to insulin-like growth factor I.**M.A. Guvakova¹, I. Prabhakaran¹, W.S. Lee¹, D.K. Furstenau¹, D.C. Li¹, R. Hung¹, N. Kushnir¹;¹University of Pennsylvania, Philadelphia, PA

How the Ras-related GTPase Rap1 controls a fine balance between cell adhesion and cell migration remains puzzling. Here, we discovered a previously unidentified regulatory role of insulin-like growth factor type I receptor (IGF-IR) in CRK SH3-binding guanine nucleotide exchange factor (C3G)-Rap1-fascin-actin axis promoting cell movement. We demonstrate that a burst of Rap1 activity, rather than presumed hyperactivation is imperative for the onset of cell movement. We show that while autophosphorylated IGF-IR signals to C3G to activate Rap1, subsequent IGF-IR internalization promotes gradual inactivation of Rap1 by putative Rap1GTPase-activating protein (GAP). Additionally, IGF-IR signaling recruits active Rap1 at sites of cell motile protrusions. C3G depletion prevents IGF-I-induced accumulation of fascin at actin microspikes and blocks protrusions. In the absence of IGF-IR's activity, the wild type Rap1 and the constitutively active V12Rap1 mutant remain in cell-cell contacts. Forced inactivation of Rap1 signaling by overexpressing dominant negative N17Rap1, Rap1GAP, or by silencing C3G has a detrimental effect on F-actin and cell adhesion irrespective of IGF-IR signaling. We conclude that the basal levels of Rap1 activity hold up cell adhesion, whereas sequential regulation of C3G and GAP by IGF-IR reverses the labile Rap1 function from supporting adhesion to promoting migration. Furthermore, our findings support a role of C3G-Rap1 signaling in the IGF-IR-mediated regulation of an actin crosslinking protein fascin.

P73**Board Number: B173****Human formin-2 generates nuclear actin filaments in response to DNA damage.**B.J. Belin¹, T. Lee¹, R.D. Mullins²;¹Cellular Molecular Pharmacology, University of California, San Francisco, San Francisco, CA,²Department of Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, CA

Compelling recent evidence has implicated nuclear actin filaments as regulators of cellular responses to certain environmental stresses, including serum stimulation, heat shock and cultured models of neurodegeneration (Baarlink et al., 2013; Bamburg et al. 2010). Using human tissue culture lines, we have observed that nuclear actin polymerization is stimulated by DNA damage induced by a variety of mechanisms, including telomere uncapping, UV exposure and MMS treatment. DNA damage-associated nuclear filaments can be visualized by phalloidin staining and are detected with high specificity using our nuclear actin-specific filament reporter, Utr230-EGFP-NLS. Using this tool, we identify several classes of

nuclear actin filaments after DNA damage: nucleolus-associated, nucleoplasmic and aggregate-like filament bodies. Unlike previously described nuclear actin filaments, DNA damage-induced filaments do not contain cofilin nor require mDia1/mDia2 for polymerization. Rather, we find that DNA damage-associated nuclear actin polymerization requires formin family actin nucleator formin-2 (FMN2). FMN2 accumulates in the nucleus in response to DNA damage, and FMN2 or importin-9 knockdowns implicate nuclear actin polymerization in efficient double strand break clearance and regulation of nuclear oxidation during the DNA damage response. These results suggest that regulation of nuclear actin assembly may be a common feature of cellular stress responses and reports the first characterized function for FMN2 in mammalian somatic cells.

P74

Board Number: B174

Comparative analysis of tools for *in vivo* detection of actin filaments.

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Several tools are now available for *in vivo* imaging of actin filaments, including Lifeact, variants of the utrophin actin-binding domain (Utr230 and Utr261), F-tractin and fluorescently labeled actin. Use of these filament reporters has become widespread and, despite growing evidence to the contrary, they are often regarded as comprehensive labels for actin filaments. To systematically evaluate their suitability for detecting commonly studied actin filament networks, we generated stable cell lines for each reporter in human, *Mus musculus*, *Xenopus laevis* and *Drosophila melanogaster* tissue culture cells. Within each line, we quantitatively compared reporter localization to phalloidin staining and used FRAP to determine how reporter binding and dynamics relate to filament turnover kinetics. Using this approach, we identified subtle reporter binding preferences that are qualitatively similar across organisms and provide reporter recommendations for studies of multiple filament networks, including cortical actin, lamellae, lamellipodia, filopodia and stress fibers. We find that F-tractin is the most broadly suitable filament reporter yet induces organism-specific morphological defects. Poor Lifeact binding and GFP-actin incorporation is common among formin-generated filaments, whereas Utr261 is often excluded from the Arp2/3-generated lamellipod. Utr230 is restricted to highly stable actin filaments and, surprisingly, detects Golgi-associated filaments in *D. melanogaster* S2 cells, which have previously been detected by immunofluorescence but cannot be seen with phalloidin. Overall our results underscore the need to test multiple visualization strategies to determine the most appropriate filament visualization tool for each desired application.

P75**Board Number: B175****Formin tails enhance processive elongation of actin filaments in addition to nucleation and filament bundling.**C.L. Vizcarra¹, B. Bor², M.E. Quinlan^{1,2};¹Chemistry and Biochemistry, University California-Los Angeles, Los Angeles, CA, ²Molecular Biology Institute, University California-Los Angeles, Los Angeles, CA

Formins are multi-domain proteins that assemble actin in a wide variety of biological processes. They both nucleate and remain processively associated with growing filaments, in some cases accelerating filament growth. The well-conserved Formin Homology 1 and 2 domains were originally thought to be solely responsible for these activities. Recently a role in nucleation was identified for the Diaphanous Autoinhibitory Domain (DAD), which is C-terminal to the Formin Homology-2 domain. The C-terminal tail of the *Drosophila* formin Cappuccino (Capu) is conserved among FMN formins but distinct from other formins. It does not have a DAD domain. Nevertheless, we find that Capu-tail plays a role in filament nucleation similar to that described for mDia1 and other formins. Building on this story, replacement of Capu-tail with DADs from other formins tunes nucleation activity. Capu-tail has low affinity interactions with both actin monomers and filaments. Removal of the Capu-tail reduces both actin filament binding and bundling. Further, when the tail is removed, we find that processivity is compromised. Despite decreased processivity, the elongation rate of filaments is unchanged. Again, replacement of Capu-tail with DADs from other formins tunes the processive association with the barbed end, indicating that enhancement of processivity is a general role for formin tails. Our data show a role for the Capu-tail domain in assembling the actin cytoskeleton, largely mediated by electrostatic interactions. Despite the absence of a DAD domain, the Capu-tail mimics all known functions of other DAD domains including autoinhibition. Finally, the role of formin tails in processivity was previously unrecognized.

P76**Board Number: B176****Mechanism of IRSp53 Inhibition by 14-3-3.**D.J. Kast¹, R. Dominguez¹;¹Physiology, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA

Filopodia are actin-rich, dynamic membrane protrusions that play essential roles in numerous cellular processes such as cell migration, phagocytosis, and axonal guidance. IRSp53 couples signaling pathways to membrane and cytoskeleton dynamics, acting as a potent inducer of filopodia. IRSp53 contains an N-terminal inverted BAR domain, followed by overlapping Cdc42/SH3-binding sites within a sequence known as the CRIB-PR domain, and an SH3 domain that recruits several cytoskeletal effectors. We have previously shown that activation of IRSp53 from an autoinhibited state requires the binding of Cdc42 to the CRIB-PR and/or effectors, such as Eps8, to the SH3 domain, which produce a conformational change in IRSp53. The sequence between the CRIB-PR and SH3 domains is rich in Ser and Thr residues, and

some of which bind 14-3-3 in a phosphorylation-dependent manner, providing an alternative route for IRSp53 regulation. Here we explore the phosphorylation-dependent mechanism of IRSp53 inhibition by 14-3-3. Using isothermal titration calorimetry we show that 14-3-3 binds weakly to single phosphorylated sites, but that binding is enhanced ~100-fold when specific pairs of sites are phosphorylated. Crystal structures show how the different phosphorylation sites of IRSp53 bind to 14-3-3, either as mono- or double-phosphorylated peptides. The results identify the most likely sites for inhibition by 14-3-3, and the kinases likely involved in phosphorylation of these sites. The data support a model of inhibition whereby asymmetric binding of 14-3-3 to two distinct phosphorylation sites inhibits activation by either Cdc42 or downstream effectors.

P77

Board Number: B177

Disabled regulates Abelson nonreceptor tyrosine kinase localization and kinase activity in *Drosophila*.

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Disabled (Dab) is a putative adaptor protein that is required for lamination of the cortex. In *Drosophila* it interacts with the Abelson (Abl) kinase signaling pathway to regulate epithelial structure and axon guidance. Previous evidence hints at Dab regulation of both Abl localization and Abl kinase activity. *Dab* mutant embryos show localized inhomogeneity in Abl localization during the midcellularization stage in early embryogenesis. Disabled structure, such as its polyproline domains which are potential SH3 binding regions, and the PTB domain, suggests that Dab may act as a scaffold and can potentially recruit Abl, by binding it directly or in a complex, may regulate its ability to bind to other proteins. We initiated a structure-function analysis to determine what domains of Dab may regulate Abl localization and activity using tagged Dab deletion constructs transiently transfected into S2 cells and subsequently assayed for Abl localization by immunofluorescence and confocal microscopy. To assay Abl activity, we took advantage of a FRET based Abl kinase reporter. We found that overexpression of Dab full length or Dab constructs containing the highly conserved central domain aggregate in puncta in S2 cells and these puncta colocalize with puncta of endogenous Abl, suggesting Dab recruitment of Abl in the cytoplasm. Under these conditions upon overexpression of Dab, we found increased Abl kinase activity as assayed by FRET, suggesting that Dab stimulates Abl kinase activity. We have further confirmed this increase in Abl kinase activity via FRET *in vivo* in cultured photoreceptors from *Drosophila* larvae overexpressing Dab. Conversely, *dab* mutant larvae have reduced Abl kinase activity as assayed by FRET. In conclusion, we have found that in S2 cell culture Dab adaptor protein regulates Abl kinase localization and activity. In the future we will make further refined deletions of Dab to determine what protein domains are responsible for these activities.

P78**Board Number: B178****Dendritic actin network growth in the absence of symmetry breaking.**P. Jreij¹, P. Bieling^{1,2}, T. Li¹, R.D. Mullins², D.A. Fletcher^{1,3,4};¹Department of Bioengineering, University of California, Berkeley, Berkeley, CA, ²Department of Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, CA, ³Biophysics Graduate Group, University of California, Berkeley, Berkeley, CA, ⁴Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA

At the leading edge of migrating cells, dendritic actin networks exert protrusive forces through the dynamic machinery of Arp2/3-mediated actin polymerization. Previous studies have shown that dendritic actin network assembly can be reconstituted on nucleation promoting factor (NPF)-coated surfaces from a minimal system of purified proteins including actin, profilin, Arp2/3, and capping protein. Variation in concentrations of these components has produced insight into their biochemical interplay and resulting network structure. However, in most studies to date, the requirement for actin networks growing around beads or bacteria to break symmetry before exhibiting steady-state growth introduces non-uniform stresses that could alter network structure and has limited the range of protein concentrations that could be explored. To investigate a broader range of protein concentrations and avoid stresses due to curved surfaces, we reconstituted dendritic actin networks on planar glass surfaces using silane-PEG-maleimide chemistry to pattern NPF APVCA domains on defined areas. In the presence of actin, profilin, Arp2/3, and capping protein, branched actin networks assembled on the patterns and grew out of plane at a constant rate. We followed the dynamic growth in real time using three-color spinning disk confocal microscopy for a range of NPF densities, Arp2/3 concentrations, and capping protein concentrations. Our findings show that the net rate of actin incorporation into the network is independent of changes in NPF density, Arp2/3 concentration, and capping protein concentration, even though network growth rates vary significantly in response to these changes. Our estimates of nucleation rates, filament lengths, and changes in number of free barbed ends across a range of Arp2/3 and capping protein concentrations are consistent with the Monomer Gating Model and help to constrain predictive models of dendritic actin network growth.

P79**Board Number: B179****Isoform-specific oligomerization of ZASP, a myofibrillar myopathy gene product, in vitro and in vivo.**R. Ohman¹, L. Brubaker¹, S. Kawakami-Schulz¹, X. Lin¹, K. Subedi¹, I. Chow¹, J. Kaufman², P. Wingfield², A.K. Mankodi¹;¹NINDS-NIH, Bethesda, MD, ²NIAMS-NIH, Bethesda, MD

BACKGROUND: Myofibrillar myopathies (MFM) present clinically as progressive muscle weakness that begins in early adulthood. Zaspopathy, a prototype MFM, is caused by heterozygous mutations in ZASP,

a Z-disc associated PDZ-LIM protein. The long and short ZASP isoforms are defined by the presence or absence of LIM domains, respectively. The mutated sZM domain is common to both isoforms. ZASP interacts with skeletal actin filaments via the sZM domain. The PDZ-LIM proteins are regulated by intramolecular association. However, oligomerization of ZASP has not yet been explored. OBJECTIVE: 1) To explore self-association of ZASP; 2) To identify oligomerization domains of ZASP; and 3) To investigate the role of ZASP self-association in normal skeletal muscle and in zaspopathy. DESIGN/METHODS: ZASP self-association was investigated with analytical sedimentation equilibrium assay of purified ZASP proteins as well as co-immunoprecipitation and chemical crosslinking assays in transfected cells and mouse and patient skeletal muscle. The domains involved in dimerization were identified with yeast two-hybrid assays. Actin-bundling ability of ZASP was explored using a low-speed co-sedimentation assay. Effects of different ZASP isoforms on actin stress fibers were examined in cultured mouse muscle (C2C12) cells. RESULTS: The long ZASP isoforms self-associate, whereas the short isoform does not. Wild type and mutant ZASP form homodimers and heterodimers. ZASP self-association occurs via interaction of the LIM domains. The mutated actin-binding domain is not involved in ZASP self-association. A low-speed co-sedimentation assay showed that GST or His6-tagged long ZASP isoform does not crosslink actin filaments. Expression of the mutated ZASP long but not short isoform caused F-actin disruption in cultured muscle cells. CONCLUSIONS: Wild-type and mutant ZASP form homodimers and heterodimers in zaspopathy, an autosomal dominant disease. Self-association of ZASP and F-actin disruptive properties of mutated ZASP are specific to the long isoform that is required for the integrity of the Z-discs in skeletal muscle. Future studies will explore the effects of post-translational modification on ZASP dimerization. Understanding ZASP associations within both the normal and disrupted cells of the Z-disc will lead to a better understanding of structural components of the Z-disc, and the contribution of disruption of ZASP-actin associations in MFM.

Regulation of Actin Dynamics 1

P80

Board Number: B180

Counting actin molecules in cytoskeletal structures with Lifeact by quantitative fluorescence microscopy.

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Quantitative measurement of actin molecules in cytoskeletal structures is essential to understand many cellular processes, but counting actin directly using fluorescence microscopy has been challenging, because actin tagged with a fluorescent protein such as mGFP is not fully functional and cannot replace native actin. Here we present a method using Lifeact-mGFP to count actin molecules in contractile rings and actin patches in live fission yeast cells. Purified Lifeact-mCherry binds actin filaments stoichiometrically with a Kd of 16 μ M. To titrate actin filaments in fission yeast we drove the expression of Lifeact-mGFP with an inducible promoter 3nmt1 and obtained cells with a wide range of total fluorescence. We used quantitative fluorescence microscopy to measure the number of Lifeact-mGFP molecules in whole cells and actin patches and contractile rings in these cells. The total numbers of Lifeact-mGFP associated with both structures increased with the cytoplasmic concentration and plateaued at high concentrations giving an apparent Kd of about 20 μ M. From the plateaus of these binding curves we estimated a peak number of ~5,000 actin molecules in actin patches (similar to published values with actin-mGFP) and ~150,000 actin molecules in complete cytokinetic contractile rings.

P81

Board Number: B181

In vivo system for identifying nuclear actin regulators.

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Actin is not only a critical component of the cytoplasmic cytoskeleton, but plays key roles in the nucleus – including chromatin remodeling, transcription and nuclear organization. However, the structures,

functions, and how structure affects function of nuclear actin remain poorly understood. While nuclear actin does not typically form canonical filaments as observed by phalloidin staining, other unique structures have been observed. One of these structures is nuclear actin rods. Actin rods form in response to cellular stress (i.e. heat shock, starvation/re-feeding), and when cytoplasmic filamentous actin is disrupted. Nuclear actin rods are also observed during the progression of neurodegenerative diseases including Alzheimer's and Parkinson's disease. While nuclear actin rods have been implicated in contributing to cell survival, their functions and the mechanisms regulating their formation remain largely unclear. We have identified a new, in vivo, multicellular system to study nuclear actin rods. Ectopic expression of GFP-Actin results in nuclear actin rod formation within the nurse cells during stages 5-9 of *Drosophila* oogenesis. Rod formation depends on which of the six *Drosophila* actins is overexpressed and its level of expression. Notably, endogenous nuclear actin is observed in the same cells during these developmental stages, suggesting that nuclear actin plays a functional role during egg development. Thus, we can utilize the actin rods formed when GFP-actin is overexpressed as a system to uncover conserved means of regulating nuclear actin. Specifically, we are quantitatively assessing the prevalence and morphology of the actin rods formed by the different actins, and how those rod phenotypes are altered by cellular stress. We are also identifying novel mechanisms regulating nuclear actin rod formation and/or structure by genetically screening for phenotypic modifiers. These studies are expected to provide significant insight into the regulation and function of nuclear actin not only during development but also during neurodegeneration.

P82

Board Number: B182

Tropomyosin 5a/b actin filaments collaborate with arp2/3 actin filaments to regulate cell motility.

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The mammalian actin cytoskeleton is differentially regulated by over 40 different isoforms of tropomyosin. Different isoforms sort to spatially distinct actin filaments within the cell where they perform isoform-specific regulatory roles. Using isoform-specific antibodies, we have demonstrated that of all known isoforms of tropomyosin, only Tm5a and 5b are enriched in the lamellipodium of mouse fibroblasts, indicating a role in the regulation of actin filament function within this structure. This led us to further investigate the functional role of these isoforms in the regulation of plasma membrane protrusions associated with cell motility. Using live-cell imaging, kymography and cell tracking we have shown that knockdown of Tm5a/b expression through RNA interference leads to a marked decrease in the persistence of membrane protrusions but no change in the level or location of arp2/3. This indicates that arp2/3 location is not dependent on Tm5a/b. A corresponding reduction in whole cell motility is observed in Tm5a/b knockdown cells which is paralleled by a reduction in the number of substratum adhesions. These findings suggest that the actin cytoskeleton within the lamellipodium contains two

types of actin filaments with different functional roles. One is the well-characterised branched filament network driven by arp2/3 which provides the protrusive force to generate dynamic membrane protrusions. The second is Tm5a/b-containing actin filaments, most likely unbranched, which stabilise arp2/3 protrusions by promoting the formation of adhesions to the substratum to gain traction. We propose that tropomyosin behaves as a discriminating factor that allows collaboration of different filament types to achieve a specific functional outcome for the cell.

P83

Board Number: B183

Nucleation of Actin Filaments by the Arp2/3 Complex: New Mechanistic Insights from Kinetic Simulations.

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The actin related protein 2/actin related protein 3 (Arp2/3) complex is a widely conserved actin nucleation factor. The Arp2/3 complex controls actin dynamics by nucleating new actin filaments in specific places, times and orientations in the cell, in response to stimulatory signals from nucleation promotion factors (NPFs) such as those of the WASP family. The fundamental signaling logic has been understood for several years; coincident signals from NPFs and pre-existing filaments combine to stimulate Arp2/3 complex to produce new filaments. Given the central role that Arp2/3 complex plays in many processes, there has been great interest in the detailed mechanism by which it nucleates new actin filaments. Here we use computational simulation of the kinetics of the nucleation process to explore a recent hypothesis that NPF release from the complex is required during nucleation.

While NPFs do promote high levels of activation, structures of actin in complex with a necessary actin binding NPF element, the WASP Homology 2 (WH2) region, indicate that their engagement with actin during delivery may prevent growth of the nucleated filament. This idea is consistent with recent single molecule studies. Here we incorporate this idea into a new kinetic mechanism that also enforces thermodynamic reversibility and allosteric coupling. By comparing the predicted behavior of this model to the global behavior of in vitro Arp2/3 complex nucleation activity, we find that agreement with the data is improved but requires additional kinetic steps for a complete description. Systematic exploration of parameter space for a series of successively more complex models has lead to a new kinetic model of Arp2/3 complex activation by NPFs. This new model will serve as a predictive template to guide further studies refining our understanding of this essential nucleation factor.

P84**Board Number: B184****Collapsin Response Mediator Protein-1 as a novel factor modulating Arp2/3-dependent actin structures.**H. Yu-Kemp¹, W.M. Brieher¹;¹Cell and Developmental Biology, University of Illinois-Urbana-Champaign, Urbana, IL

Listeria monocytogenes is a parasite that uses host proteins to assemble an Arp2/3-dependent actin comet tail for its propulsion. Comet tail assembly is more efficient in cytosol than in defined systems implying that unknown factors contribute to this reaction. We fractionated bovine brain cytosol and identified Collapsin Response Mediator Protein-1 (CRMP-1) as such a factor. CRMP-1 has long been studied as a factor for microtubule dynamics. Here, we show that CRMP-1 contributes to Arp2/3-dependent actin dynamics. Immunodepletion of CRMP-1 from brain cytosol reduced Arp2/3-dependent actin assembly around *Listeria*, and the defect could be successfully rescued with recombinant CRMP-1. In vitro assays confirmed that CRMP-1 enhanced Arp2/3-dependent branching and stimulated ActA- as well as VCA-activated Arp2/3-dependent actin polymerization. To test the contribution of CRMP-1 to Arp2/3-dependent polymerization inside cell, we examined the localization of CRMP-1 using immunostaining and GFP-CRMP-1 overexpression in epithelial cells. CRMP-1 localized to the leading edge of lamellipodia and cadherin-mediated cell-cell contacts, both of which are known to be Arp2/3-dependent structures. Knockdown and overexpression approaches revealed a positive correlation between the amount of CRMP-1 and the amount of F-actin inside the cell. In wound closure assays, CRMP-1-depleted cells had twice less protrusions at the wounded edge, with half actin intensity at the leading edge compared to control cells; while overexpression of CRMP-1 provided the opposite results. During wound closure, CRMP-1-depleted cells moved erratically and lost their directionality with unstable membrane protrusions. In contrast, GFP-CRMP-1 overexpressing cells moved unidirectionally, with the membrane at the leading edge stably protruding forward. Depletion of CRMP-1 also reduced the amount of cadherin and actin at cell-cell contacts, resulting in weaker cell adhesion for epithelial cells. Immunoblotting results indicated CRMP-1 present in the membrane fraction of cadherin enriched plasma membrane. This membrane had been shown to be able to assembly actin filaments in an Arp2/3-dependent manner. An antibody against CRMP-1 blocked this Arp2/3-dependent reaction, showing the role of CRMP-1 on regulating Arp2/3-dependent actin assembly at cadherin-mediated cell-cell contacts. In summary, our results identify CRMP-1 as a novel regulator of the actin cytoskeleton that specifically contributes to Arp2/3-dependent actin assembly. CRMP family proteins have been studied in regulating metastasis and neuronal growth cone guidance. Our data provide a mechanism for understating how CRMP proteins might modulate those biological events through their potential role in actin cytoskeletal organization.

P85**Board Number: B185****Isoform diversity modulates the ability of the Arp2/3 complex to stimulate actin polymerization.**J.V. Abella¹, C. Galloni¹, M. Howell², M. Way¹;¹Cell Motility Group, London Research Institute - Cancer Research UK, London, UK, ²High Throughput Screening Laboratory, London Research Institute, London, United Kingdom

The Arp2/3 complex is unique among actin nucleators in its ability to generate branched actin filament networks. These networks are critical in maintaining the architecture of the cell and regulating cell motility, invasion, endocytosis and phagocytosis. A number of intracellular pathogens, such as *Listeria*, *Shigella* and *Vaccinia* virus also use the Arp2/3 complex to stimulate actin polymerization to enhance their cell-to-cell spread. The Arp2/3 complex consists of 7 subunits (Arp2, Arp3 and ARPC1-5), which are evolutionary conserved from protozoa to mammals. Interestingly, in humans, Arp3, ArpC1 and ArpC5 have two isoforms that are 91, 67 and 67% identical respectively. This raises the possibility that Arp2/3 complexes with different properties may exist. We found that the each isoform of Arp3, ARPC1 and ARPC5 is incorporated into an Arp2/3 complex and recruited to vaccinia induced actin tails. However, specific combinations differentially regulate actin-based motility of the virus. Depletion of ARPC1A and ARPC5, individually or together, results in longer actin tails. Conversely, loss of ARPC1B and/or ARPC5L results in very short actin tails. Loss of Arp3 versus Arp3B also results in opposite actin-tail phenotypes. Importantly, we demonstrate that isoform-mediated differences in actin tail length are dependent on both the rates of actin polymerization and filament disassembly. FRAP analysis, however, reveals that recruitment and turnover of each isoform on the virus is identical. This suggests that the activity of the Arp2/3 complex depends on its subunit composition. These differences are not unique to vaccinia-induced actin polymerization. For example ARPC5L but not ARPC5 is essential for invadopodia formation and gelatin degradation. Moreover, Arp2/3 complex subunit composition also impacts on the rate of cell migration. Our observations now demonstrate that the Arp2/3 complex can no longer be considered as a unique functional assembly.

P86**Board Number: B186****Coronin 7 and its role in organizing the actin cytoskeleton.**K. Bhattacharya¹, K. Swaminathan², A. Noegel^{1,3,4,5}, R. Rastetter⁶;

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Coronins are F-actin binding proteins which belong to an evolutionary conserved family of WD40-repeat proteins. They are widely expressed in cells and tissues and are involved in signal transduction, transcriptional regulation, remodeling of the cytoskeleton and regulation of vesicular trafficking. Here, we focus on mammalian Coronin 7 (CRN7), the only 'long' coronin known in mammals, having two WD40-repeat domains which form a highly symmetrical tandem β -propeller. CRN7 localizes to the Golgi apparatus where it has a role in protein trafficking and maintenance of Golgi morphology. However, the role of CRN7 in cytoskeleton remodeling has not been addressed so far. Therefore, we have generated a CRN7 knockout (KO) mouse model and established primary fibroblast cultures to address the cellular functions and regulatory mechanisms involved with respect to the dynamics of the actin cytoskeleton.

Analysis of CRN7 KO cells in vitro revealed that primary CRN7 KO fibroblasts exhibited a disrupted Golgi architecture and altered Golgi-centrosome positioning compared to the wild type (WT) counterpart. Importantly, fibroblasts deficient of CRN7 exhibited a faster cell polarization, an increased cell migration and an accelerated wound closure. Additionally, we detected a higher F-actin content in the KO fibroblasts. Re-expression of CRN7 reversed the Golgi-related phenotypic deficits in the KO fibroblasts.

In line with the recently published data that a Cdc42- and Rac-interactive binding (CRIB) domain mediates functions of coronin (*corA*) in *Dictyostelium discoideum*, we likewise study here the significance of the CRIB motif present in CRN7 on both β -propellers. We found that CRN7 has a higher affinity for GDP-bound GTPases than for the corresponding GTP-bound GTPase and therefore, we draw our focus on investigating the downstream effectors of CRN7-mediated regulation of small RhoGTPases. Taken together, this study will provide an insight into the role of mammalian CRN7 as an organizer of actin-dependent cellular events.

P87**Board Number: B187****Coronin organizes actin filament polarity required for directional gliding motility in the malaria parasite.**

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Actin-dependant processes such as cell motility rely on the appropriate control and regulation of dynamic filamentous actin. Coronins are a conserved family of regulators found across all eukaryotes and are involved in the coordination of F-actin dynamics in motile cells. Different coronins display varying functions, including binding directly to F-actin and crosslinking filaments, modulating F-actin assembly and branching via interactions with the Arp2/3 complex, as well as by mediating F-actin disassembly by coordinating ADF/cofilins. Many eukaryotic genomes encode several types of coronins, while others such as *Plasmodium* encode only one. The entire actin regulatory network in *Plasmodium* is drastically reduced, consisting of only a handful of members, and strikingly no Arp2/3 complex. Here we determine the function of *Plasmodium* coronin in the human and murine malaria parasites *Plasmodium falciparum* and *P. berghei*. We demonstrate that *Plasmodium* coronin is able to bind and organize actin filaments in vitro into parallel-bundled actin cables and bind to the membrane phospholipid phosphatidylinositol (4,5) bisphosphate. In vivo *Plasmodium* coronin localises to the cell periphery and relocalizes during actin-dependent fast movement. Finally, knockout of coronin in *P. berghei* results in aberrant fast movement altering mosquito-stage sporozoite movement from circular to erratic. These combined data suggest *Plasmodium* coronin is a critical part of the organizing architecture of directional force generation required by malaria parasite motility.

P88**Board Number: B188****PI(3,5)P2 controls branched actin dynamics at late endosomes by regulating cortactin-actin interactions.**N. Hong¹, A.M. Weaver^{1,2};¹Department of Cancer Biology, Vanderbilt University Medical Center, Nashville, TN, ²Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, TN

Branched actin critically contributes to membrane trafficking by regulating membrane curvature, dynamics, fission and transport. However, how actin dynamics are controlled at membranes is poorly understood. Here, we identify the branched actin regulator cortactin as a direct binding partner of the phosphoinositide (PI) PI(3,5)P2 and demonstrate that their interaction promotes turnover of late endosomal actin assemblies. In vitro biochemical studies indicate that cortactin binds PI(3,5)P2 via the actin filament-binding region of cortactin. Furthermore, actin filaments and PI(3,5)P2 compete for binding to purified cortactin, suggesting that formation of PI(3,5)P2 on endosomes may remove cortactin from endosome-associated branched actin networks. Indeed, inhibition of PI(3,5)P2 production led to accumulation of both cortactin and actin on Rab7-positive endosomes. Knockdown of cortactin reversed inhibitor-induced actin accumulation at Rab7-positive endosomes. These data suggest a model in which PI(3,5)P2 binding removes cortactin from late endosomal branched actin networks and thereby promotes net actin turnover.

P89**Board Number: B189****The small GTPase ARF1 regulates podosome assembly.**N.B.M. Rafiq^{1,2,3}, Z. Z. Lieu¹, C. Yu¹, A. Krishnasamy¹, G. E. Jones³, A.D. Bershadsky^{1,2,4}; ¹Mechanobiology Institute, National University of Singapore, Singapore 117411, Singapore; ²Department of Biological Sciences, National University of Singapore, Singapore; ³Randall Division of Cell & Molecular Biophysics, King's College London, London SE1 1UL, UK; ⁴Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot 76100, Israel**The small GTPase ARF1 regulates podosome assembly**Nisha Bte Mohd Rafiq^{1,2,3}, Zi Zhao Lieu¹, Cheng-han Yu¹, Anitha Krishnasamy¹, Gareth E. Jones³, Alexander D. Bershadsky^{1,2,4}¹Mechanobiology Institute, National University of Singapore, Singapore 117411, Singapore²Department of Biological Sciences, National University of Singapore, Singapore³Randall Division of Cell & Molecular Biophysics, King's College London, London SE1 1UL, UK

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Podosomes are integrin-mediated adhesion structures often formed by cells of the monocytic lineage, as well as by other cell types, including Src-transformed fibroblasts, and play an important role in cell migration and matrix degradation. Podosomes are multiple micron-sized radially symmetrical structures consisting of a central actin core associated with an “adhesive ring” containing the integrins and associated plaque proteins. Actin polymerization mediated by Arp2/3 complex is critically important for podosome formation, while upstream signaling pathways involving Rho GTPases (in particular Cdc42), are known, otherwise they are poorly understood.

Here, we demonstrate that small GTPase ARF1, known to regulate the trafficking of COPI vesicles, is a potent regulator of podosome dynamics. We showed that down-regulation of ARF1 by siRNA led to a striking reduction in the number of podosomes in macrophage-like THP1 cells. In parallel, treatment of cells with Brefeldin A (BFA), a drug that targets a subset of Sec7-type GTP exchange factors (GEFs) of Arf1 led not only to dissociation of the Golgi apparatus but also to loss of podosomes in a dose-dependent manner. Podosome “rosettes” often observed in Src-transformed fibroblasts were also shown to undergo gradual disruption after addition of BFA. Furthermore, we found that inhibition of another subclass of ARF GEFs, cytohesins, by a small-molecule inhibitor SecinH3 led to disassembly of podosomes in both THP1 cells and Src-transformed fibroblasts. Accordingly, RNAi silencing of ARNO (Cytohesin-2), but not Cytohesin-1, led to a dramatic reduction in podosome number. Even though bulk of ARF1 is localized to Golgi apparatus, TIRF microscopy of fluorescently-tagged ARF1 revealed punctate structures localized to regions of podosome assembly; these ARF1 puncta were also co-localized with Rab11-positive vesicles. Remarkably, ARNO was found to co-localize with components of adhesive ring in individual podosomes. Finally, in immunoprecipitation experiments, ARF1 was found to interact with the Arp2/3-inhibitory protein, arpin, which in turn was shown to co-localize with the actin core of podosomes. We conclude that ARNO-ARF1 signaling is strongly involved in the regulation of podosome assembly, most probably via modulation of Arp2/3-dependent actin polymerization.

P90

Board Number: B190

Solo plays regulatory role in astrocyte migration through activation of both Rac1 and RhoA.

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Astrocytic migration plays a crucial role both in normal fetal development as well as in abnormal astrogliosis and traumatic spinal cord injury. Rho GTPases, specifically Rac1 and RhoA, have been shown to be crucial to normal astrocyte migration though signaling nuances have yet to be elucidated. GTPase activity is spatiotemporally regulated through function of guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) to activate or inactivate GTPases respectively. One such GEF, Solo, has been described as a membrane associated Trio isoform involved in neurite elongation (Aoki,

2006) and has been shown to be upregulated during mouse astrocyte migration. While Trio, the rat and human isoform, contains both GEF1 (activates Rac1 and RhoG) and GEF2 domains (activates RhoA), Solo contains only the GEF1 domain. Preliminary data indicates that Solo preferentially co-localizes with Rac1, and that siRNA Solo knock down induces altered astrocyte migration and cellular morphology. The interaction of Solo with both Rac1 and RhoA could indicate a more complex role in astrocytic migratory signaling than previously thought.

P91

Board Number: B191

Rho GTPase effector targeting during single cell wound healing.

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Wounding of single cells triggers formation and closure of an actomyosin ring around the wound. Formation of this ring reflects activation of the small GTPases, Rho and Cdc42, in concentric zones around the wound. However, the links between GTPase zones and the cytoskeleton are not well characterized. Here I have connected the Rho GTPases and cytoskeleton through analysis of the localization of Rho GTPase effectors at wounds. To determine the localization of effectors, *Xenopus laevis* oocytes were injected with mRNA encoding fluorescently labeled effectors and markers for active Rho/Cdc42 and were imaged using confocal microscopy and laser wounding. To analyze the localization patterns of effectors a new method was used to create circle scans, which are a modified line scan that encompasses 360 degrees of zone information. While some effectors such as P21 activated kinase 2 (Pak2) localize throughout the entire Cdc42 zone during wound healing, other effectors change localization over time towards the leading edge of the Cdc42 zone as healing progresses. Neural Wiskott-Aldrich syndrome protein (NWASP), which activates the Arp2/3 complex to nucleate branched actin filaments, and Transducer of Cdc42 dependent actin assembly 1 (Toca1), which is necessary for full activation of NWASP, show a slight change in localization over time towards the leading edge of the Cdc42 zone. Slingshot Phosphatase, which activates cofilin to sever actin filaments, localizes to the leading edge of the Cdc42 zone to a greater extent than NWASP or Toca1. Eliminating the ability of Toca1 to bind Cdc42 inverts the localization of Toca1 such that it localizes to the trailing edge and outside of the Cdc42 zone instead of the leading edge. Cofilin shows the most prominent shift in localization over time towards the leading edge of the Cdc42 zone. LIM kinase (LIMK1), a Rho and Cdc42 effector that inhibits cofilin, localizes throughout the Rho and Cdc42 zones with a peak in the Rho zone. Formin-like 1, an unbranched actin nucleator, localizes throughout the Cdc42 zone and in the Rho zone. Rho associated kinase (ROCK), a Rho effector that activates Myosin 2, localizes to the Rho zone. The fact that different effectors show different patterns of localization during wound healing suggests that there is subcompartmentalization within a Rho GTPase zone. This ultimately leads to the idea that similar to gradients of information leading to morphogenesis, a single cell is also capable of pattern formation using gradients of information to close a wound.

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Board Number: B192

Mechanosensitivity of actin assembly maintains homeostasis in the stress fiber network.S. Wang¹, S. Thiyagarajan¹, M.A. Smith², E. Blankman², L.M. Chapin², M.C. Beckerle², B. O'Shaughnessy³;¹Department of Physics, Columbia University, New York, NY, ²Huntsman Cancer Institute, University of Utah, Salt Lake City, UT, ³Department of Chemical Engineering, Columbia University, New York, NY

The cytoskeleton is a complex network subject to constant remodeling. A major component is the network of actomyosin stress fibers. Even in cells at rest subject to no external perturbations, the network undergoes continuous and apparently stochastic adjustments as stress fiber sarcomeres change length in an apparently random fashion. The origin of these fluctuations, and the mechanism that cells employ to continuously remodel actin to accommodate these length changes and maintain network homeostasis are unknown.

Here we systematically measured the statistics of stress fiber fluctuations in mouse embryonic fibroblasts stably expressing zyxin-GFP and other fluorescently tagged stress fiber components. These statistics are a constant of the network and define its stochastic dynamics, even though the network itself is in constant flux. Using zyxin-GFP to identify sarcomere boundaries from time-lapse confocal microscopy images, we measured the temporal statistics of sarcomere length over many sarcomeres and stress fibers, following individual sarcomeres for ~ 1000 s. Statistical analysis revealed an early phase (less than ~ 50 s), when sarcomere length changed randomly in time with an effective length 'diffusion constant' $D_{\text{early}} \sim 1000 \text{ nm}^2/\text{s}$, and a late phase (greater than ~ 50 s) with a smaller diffusion constant $D_{\text{late}} \sim 200 \text{ nm}^2/\text{s}$. This remarkable feature was reproducible over many cells and stress fibers.

To explain this behavior we developed a mathematical model attributing the stochasticity to myosin-II, the principal driving force for sarcomere length changes. Fluctuations in myosin-II force are inevitable due to turnover, variations in phosphorylation and the fraction of heads bound to actin, etc. We hypothesized that actin disassembly (assembly) is mechanosensitive, steered by elastic stresses which grow (decrease) when sarcomeres shorten (lengthen) and actin filaments are forced to overlap (pulled apart). This mechanism quantitatively reproduced the observed behavior: (i) The early phase emerged as the initial sliding of actin filaments at fixed length as a sarcomere shortens or lengthens. This resets the degree of overlap, and the net actin assembly rate that depends on this overlap. (ii) In the late phase, filaments continuously adjust length in phase with the changing sarcomere length. Both phases show linear time dependence of the mean square change in sarcomere length, as seen experimentally. Best fit model parameters were remarkably close to those obtained from our previous study of spontaneously severing stress fibers.

Thus, mechanosensitivity of actin assembly allows cells to continuously remodel the stress fiber network and to maintain network homeostasis.

P93**Board Number: B193** **α -Actinin modulates actomyosin network contractile response.**H. Ennomani*¹, G. Letort*¹, C. Guérin¹, R. Boujemaa-Paterski¹, F. Nédélec², E. De La Cruz³, M. Théry⁴, L. Blanchoin¹;¹iRTSV, CEA, Grenoble, France, ²EMBL, Heidelberg, Germany, ³Yale University, New Haven, CT, ⁴iRTSV, CEA, Paris, France

Cellular contractility – the internal generation of force or tension by a cell orchestrated by the actomyosin machinery – has emerged as a critical regulator of a wide range of processes during development. Tremendous efforts have deepened our understanding of the biochemical and mechanical properties of the actomyosin contractile units encountered in the complex cell environment. Yet, how the structural organization of actin filaments regulates myosin-driven contraction remains to be established.

We have recently developed a micropatterning method that enables the spatial control of actin nucleation sites for in vitro assays (Reymann et al., 2010). These actin templates were used to evaluate the response of oriented actin structures to myosin-induced contractility in presence of regulatory proteins. We demonstrated that crosslinking level modulates actin network deformation and/or disassembly upon myosin-induced tension. We showed also that crosslinkers are necessary to sustain myosin-driven deformation and force production of dynamic actin networks in the presence of disassembly factors (ADF/cofilin). In addition, we developed numerical simulation in order to relate the observed myosin-driven actin deformation with the underlying microscopic mechanism.

Because we are working with a reconstituted system for which we control the composition and the geometrical conditions, we are in a very good position to study the fundamental rules by which myosin-mediated contractility produce force.

P94**Board Number: B194****Mechanisms of actomyosin contraction during embryonic wound repair in *Drosophila*.**A. Kobb¹, R. Fernandez-Gonzalez^{1,2,3};¹Institute of Biomaterials and Biomedical Engineering, University of Toronto, Toronto, ON, ²Department of Cell and Systems Biology, University of Toronto, Toronto, ON, ³Developmental and Stem Cell Biology Program, The Hospital for Sick Children, Toronto, ON

The coordination of cellular behaviors is critical during embryonic development, cancer metastasis and tissue repair. Coordinated cell movement is mediated by networks of actin and the motor protein non-muscle myosin II that span multiple cells. In particular, during embryonic wound repair, the cells

adjacent to the wound accumulate actin and myosin at the interface with the wounded cells. Actomyosin redistribution results in the assembly of a supracellular actomyosin cable around the wound whose contraction acts as a purse string and drives wound closure. However, the mechanisms of actomyosin recruitment to the wound margin and purse string contraction are not well understood.

The mechanisms of actomyosin contraction have been investigated during cytokinesis, the final step of cell division. During cytokinesis, a contractile actomyosin ring separates the daughter cells, and actin depolymerization, but not polymerization, plays a central role in the contraction of the ring. To investigate actomyosin dynamics during embryonic wound repair, we used a high-power ultraviolet laser to irradiate and create wounds in the epidermis of *Drosophila* embryos expressing fluorescently-tagged filamentous actin or myosin. We used Fluorescence Recovery After Photobleaching (FRAP) to quantify actomyosin dynamics at the wound margin and in cells far from the wound, during purse string assembly (2 min after wounding) and contraction (4 min after wounding). Our results showed that filamentous actin fluorescence rapidly recovered to 50% of its initial value within 22.5 ± 6.5 seconds during purse string assembly, and within 16 ± 4 seconds during contraction. Using image analysis we found that total actin levels at the purse string remained relatively constant throughout wound closure. Together, these data suggest that, unlike in cytokinesis, actin polymerization takes place during wound closure. Using FRAP, we found that myosin fluorescence at the purse string had a 1.5-fold greater mobile fraction compared to non-contractile myosin cables away from the wound, consistent with a mechanisms in which actomyosin filaments slide with respect to each other to promote contraction. We are currently integrating these data into a computational model to investigate actomyosin dynamics during purse string contraction. This model will allow us to make predictions about the role of different actomyosin regulators that we will then test experimentally to understand the molecular mechanisms by which cells coordinate their migration during embryonic wound repair.

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Board Number: B195

Myosin light chain kinase activity regulates front-back polarity during cell migration by modulating protrusion lifetime.

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Rapidly motile cells such as neutrophils and keratocytes typically polarize to generate a single front and back in order to sustain productive forward movement. However, other less persistently motile cell types, such as fibroblasts and epithelial cells, frequently have multiple, apparently independent, protrusive fronts. The mechanisms that limit polarization to a single front and back for rapidly motile cells, and how those mechanisms differ for slow-moving cell types are not fully understood. Here we develop embryonic zebrafish keratocytes as a model system for keratocyte cell biology, and use these cells to investigate how a purely lamellipodial cytoskeleton self-organizes to regulate the number of

protrusive fronts and overall cell polarity. Specifically, we have observed that keratocytes isolated from 4 days post-fertilization (dpf) embryos often form multiple protrusive fronts, suggesting that the motility machinery in these cells polarizes differently from single-front keratocytes such as those found at 2dpf or in adults. Using a combination of genomic, genetic, and pharmacological approaches, we identify increased myosin light chain kinase (MLCK) expression in 4dpf keratocytes as a causative agent in enabling the formation of multiple protrusions. We show that MLCK activity influences cell polarity by increasing myosin accumulation in lamellipodia, which decreases protrusion lifetime, thus limiting their size. These results suggest that rapidly motile cells may be able to polarize to a single front and back because they normally have less myosin activity than many slow-moving cell types

P96

Board Number: B200

CARMIL2 localizes to vimentin filaments and leading-edge membrane-ruffles in migrating cells.

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Although recent studies have demonstrated that vimentin can regulate lamellipodia formation, the mechanisms through which vimentin influences actin assembly are not well understood. However, CARMIL2 is a strong candidate to play a key role. Previous studies have shown that CARMIL2 localizes to vimentin filaments in cells and regulates capping protein (CP), a key regulator of actin networks, in actin polymerization assays. Additionally, we have identified a membrane-localizing region near the C-terminus of CARMIL2 that is both necessary and sufficient for localization to leading-edge membrane-ruffles and the membrane surrounding macropinosomes. CARMIL2 knockdown cells exhibit migration defects, diminished lamellipodial ruffling and macropinocytosis, and possess a striking multi-protrusion phenotype. How the CARMIL2 localization pattern relates to CARMIL2 function and the importance of CP regulation by CARMIL2 for cell migration are not established. We hypothesize that CARMIL2 regulates polarity during cell motility by regulating CP and coordinating actin filament dynamics with the vimentin network. Here, we report live-cell dynamics of CARMIL2 on vimentin filaments and membrane-ruffles. Additionally, we have created CARMIL2 vimentin-localizing mutants and CARMIL2 membrane-binding mutants. In the future, we will test the ability of these mutants to rescue the CARMIL2 knockdown phenotype.

Myosin Motors 1

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Board Number: B202

Structural determinants of myosin-I mechanosensing: the N-terminal region.

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Myosins are actin-based motors that are mechanically and kinetically tuned to function in a wide range of cellular processes. The myosin-I family members Myosin-IB (Myo1b) and Myosin-IC (Myo1c) have similar structures and ATPase kinetics. However, when mechanical loads are placed on the motors, they display very different behaviors. The ATP-dependent, actin-detachment kinetics of Myo1c are relatively unchanged when the myosin powerstroke is resisted by loads of less than 2 pN, so it is able to generate power and act as a molecular transporter. However, the ATP-dependent, actin-detachment kinetics of Myo1b slow dramatically under similar loads, enabling it to act as a tension-sensing anchor. We recently determined the high-resolution structure of the Myo1b motor domain and found the N-terminal region (NTR) in a conformation that has not yet been observed in other myosins. Because the NTR sequence is highly variable and alternatively spliced within the myosin-I family, we investigated whether the NTR plays a role in tuning the mechanochemical properties of myosin-I motors. We generated recombinant constructs of Myo1b and Myo1c in which their NTRs were deleted or swapped, and we characterized their kinetic and mechanochemical properties using stopped-flow techniques, single molecule optical trapping techniques, and in vitro actin-gliding assays. Our results show that the NTR does indeed play an important role in tuning the mechanochemical properties of Myo1b and Myo1c. Moreover, our results demonstrate differences in how the NTR tunes Myo1b and Myo1c, suggesting that the sequence diversity and alternative splicing of this region may play an important role in generating diversity of myosin-I function in the cell. This work was supported by a grant to EMO from NIH (GM057247).

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Board Number: B203

Functional analysis of disease-associated mutations in myosin I using fission yeast as a model system.

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Myosin 1e (myo1e) is a mammalian non-muscle myosin, which is expressed in the renal filtration unit (the glomerulus) in humans and mice. We have previously shown that Myo1e knockout mice have defects in renal filtration and glomerular ultrastructure that are similar to those observed in patients

with kidney disease called focal segmental glomerulosclerosis (FSGS). Recent genetic studies have also identified point mutations in the *MYO1E* gene in patients with FSGS. However, the precise relationship between the point mutations found in FSGS patients and the functional activity of myosin I remains to be determined. Using fission yeast *S. Pombe* as a versatile tool for analyzing myosin functions, we were able to directly test the effects of FSGS-associated mutations on myosin activity. Fission yeast have a single myosin I, Myo1p, which is associated with yeast actin patches, which are branched actin networks assembling at the sites of endocytosis. In the absence of Myo1p, yeast exhibit defects in cell growth under high salt and high temperature conditions and severe endocytosis defects. Taking advantage of the conservation of motor domains between human Myo1e and *S. pombe* Myo1p, we generated mutations in the Myo1p motor domain that were equivalent to FSGS-associated mutations in Myo1e. We found that these mutations in Myo1p resulted in the defects in yeast growth and endocytosis similar to those observed in the Δ myo1p strain. Fluorescently tagged Myo1p was used to examine myosin localization relative to actin patch marker, fimbrin. Mutations in Myo1p disrupted its localization to actin patches and resulted in relocation of Myo1p to membrane-associated protein complexes known as eisosomes. Finally, to assess the stability and folding of Myo1p mutants, we compared the localization of Myo1p to that of Rng3p, a member of UCS family proteins that acts as a myosin chaperone. Earlier studies have demonstrated that mutations in the Myo1p motor domain can promote recruitment of Rng3p to Myo1p, likely due to the decreased stability of the Myo1p. We hypothesized that Myo1p mutants that are nonfunctional due to defective folding would also interact with Rng3p and recruit it to Myo1p mutant containing structures.

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Board Number: B204

Role of Myo1 C-terminal domains in Myo1 localization and regulation of actin patch dynamics.

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Myosin-1 (Myo1), the sole type 1 myosin in *Schizosaccharomyces pombe*, is recruited to sites of endocytosis and its arrival is followed by Arp2/3 complex mediated branched actin assembly into structures known as actin patches. Myo1 has an N-terminal motor domain, a lever arm with two light chain binding motifs (IQ1 and IQ2), and a C-terminal cargo binding tail. The Myo1 tail consists of a phospholipid binding TH1 domain, a proline-rich TH2 domain, an SH3 protein interaction domain and a CA domain, which binds Arp2/3 complex. Previously, we found that disruption of IQ motifs led to decreased recruitment of Myo1 to endocytic sites and endocytosis defects, the severity of which correlated with the reduction in the amount of Myo1 at the endocytic site. To investigate the role of the Myo1 C-terminal domains in Myo1 localization and regulation of actin patch dynamics, we created a series of GFP-tagged and untagged myo1 mutants with increasingly long deletions starting from the C-terminus. Using genetic crosses we combined these mutants with mCherry-tagged fimbrin Fim1. We

found that deletion of CA did not have any effect on Myo1 localization and actin patch dynamics. Deletion of CA and SH3 domains resulted in a 30% reduction in peak levels of myosin compared to wild type, increased assembly time for fimbrin and a decreased efficiency of internalization. Deletion of CA, SH3 and TH2 domains further reduced myosin levels at the patch (60% reduction) with similar actin assembly and internalization defects as in SH3-CA deletion mutant. The TH2-SH3-CA deletion mutant accumulated to the same peak levels as the Myo1 mutant with an internal deletion of TH2, suggesting that the TH2 domain is a major contributor to Myo1 patch localization. Deletion of CA, SH3, TH2 and TH1 domains resulted in the loss of Myo1 localization to actin patches and endocytic internalization defects similar to those in cells with a complete myo1 deletion, suggesting that TH1 is required for Myo1 localization at the endocytic site. By crossing untagged C-terminal truncation myo1 mutants with GFP-tagged ARPC5 subunit of the Arp2/3 complex, we observed trends in patch dynamics defects that were similar to defects observed with fimbrin. However, the defects were more severe since GFP-tagged Arp2/3 complex is only partially functional and is synthetically lethal with TH1-TH2-SH3-CA truncation or complete myo1 deletion. In myo1 tail truncation mutants, similar to cells depleted for Myo1 or yeast WASp homolog Wsp1, Arp2/3 complex accumulated at the slower rate but reached the same peak levels as in wild type. Since depletion of Myo1 also reduced accumulation of Wsp1 in endocytic patches, we propose that Myo1 plays a role in recruiting Wsp1, directly or indirectly, to the endocytic sites.

P100

Board Number: B205

Characterization of kidney disease associated mutations in myosin 1e.

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Focal segmental glomerulosclerosis (FSGS) is one of the most common primary glomerular disorders that may lead to end-stage kidney disease. One of the pathological features of FSGS is the presence of massive proteinuria (protein excretion in the urine), which results from defects in protein filtration by the renal filtration unit, the glomerulus. Pathogenesis of FSGS involves impaired function of glomerular podocytes, specialized epithelial cells that cover the surface of the glomerular capillaries. Myosin 1e (Myo1e) is a non-muscle myosin, which is expressed in kidney podocytes in humans and mice. We have previously found that Myo1e is a component of the specialized junctional complexes associated with cell-cell contacts between podocytes, and that mice lacking Myo1e develop proteinuria. Mutations in the *MYO1E* gene have also been found in patients with childhood familial FSGS. Based on these findings, recent sequencing studies focused on identifying FSGS-associated mutations have included *MYO1E* as one of the genes of interest. This resulted in identification of novel mutations in Myo1e whose effects on protein localization and function have not been determined. Using immortalized Myo1e-null cultured podocytes that were isolated from knockout mouse kidneys, we were able to examine localization and activity of the mutant Myo1e constructs. We introduced GFP-tagged Myo1e constructs into cultured podocytes using adenoviral vectors and studied their localization in live cells. We have also utilized live-cell imaging to visualize the recruitment of junctional components during cell-

cell contact assembly. Using this approach, we were able to directly demonstrate the pathogenic nature of some of these mutations, which result in mislocalization of Myo1e and defects in cell-cell junction assembly. The observed junctional defects may lead to the formation of “leaky” glomeruli *in vivo*.

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Board Number: B206

Myosin 1e role in regulation of breast cancer cell migration and invasion.

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Cell migration contributes to many normal physiological processes such as embryogenesis and the immune response, but can also be pathological as seen with cancer cell invasion and metastasis. The actin cytoskeleton and its associated proteins, including myosin motors, play an important role in cancer cell migration. The aim of this study was to dissect how class I myosin 1e (Myo1e) regulates cancer cell migration. Myo1e includes a motor domain that interacts with actin filaments and a tail domain that contains membrane-binding and protein interaction motifs. Thus, it may act as a scaffold connecting actin filaments to the plasma membrane. Using an invasive breast cancer cell line MDA-MB-231 as a model, we found that shRNA-mediated knockdown of Myo1e reduced cell migration in a transwell assay compared to cells treated with scrambled shRNA. Myo1e knockdown also impaired cell invasion through Matrigel, a thin layer of matrix proteins coating a transwell filter. Observation of cells migrating towards EGF under a layer of agarose showed that control cells migrated further than Myo1e knockdown cells. Overall, these findings show that Myo1e is involved in cancer cell migration, especially in the settings where cells have to undergo complex shape changes, such as transwell migration and movement under a layer of viscous media. GFP-Myo1e localized to the outermost portion of the leading edge in migrating cells, and we are currently investigating whether it contributes to formation of protrusions at the leading edge. Based on our observations *in vitro*, we predict that Myo1e may also promote tumor cell migration *in vivo*. To test this hypothesis, we have generated Myo1e knockout mice expressing MMTV-PyMT oncogene. The MMTV-PyMT transgenic mouse is a well characterized mouse model of invasive breast cancer. We will use this model to examine how deletion of Myo1e affects breast cancer progression *in vivo*.

P102**Board Number: B207****Controlling Initiation and Termination of Kinesin-1-Driven Transport with Myosin-Ic and Non-Muscle Tropomyosin.**B.B. McIntosh^{1,2}, E.L. Holzbaur¹, E.M. Ostap^{1,3};¹Department of Physiology, University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA,²Pennsylvania Muscle Institute, Philadelphia, PA, ³Pennsylvania Muscle Institute, University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA

Intracellular transport is largely driven by processive actin- and microtubule-based molecular motors. Non-processive motors have also been localized to trafficking cargos, but their respective roles during transport are not well understood. Myosin-Ic (Myo1c) is a non-processive actin motor that functions in a variety of exocytic events, yet the molecular roles of this motor during trafficking are unclear. To investigate the interplay between myosin-I motors and the canonical long distance transport motor kinesin-1, we attached both Myo1c and kinesin-1 motors to lipid-coated bead cargo. This is a more physiological cargo-motor attachment strategy that allows motors to actively reorganize within the membrane and respond to the cytoskeletal environment. We compared the motility of these cargos in the absence and presence of Myo1c motors at engineered actin filament-microtubule intersections attached to the surface of a coverslip. We found that Myo1c influences kinesin-1 run initiation by significantly increasing the frequency of microtubule-based runs that begin at actin filament-microtubule intersections. Myo1c also regulates run termination. Beads with both motors bound consistently pause at actin-microtubule intersections, remaining tethered for an average of 20 seconds, with pauses longer than 200 seconds also observed. Actin-binding proteins such as non-muscle tropomyosin have been proposed to positively or negatively regulate the interactions between specific myosin motors and actin filament populations *in vivo*. *In vitro*, we found that non-muscle tropomyosin-2 (Tm2) abrogates Myo1c-driven actin filament gliding. In the crossed filament assay, we found that Tm2 abolishes Myo1c-specific effects on run initiation and run termination. Together these observations suggest that within the cell, Myo1c is important for the selective initiation and termination of kinesin-driven runs along microtubules at actin filament intersections. The regulation of Myo1c by tropomyosin provides a mechanism for regional specificity, preventing inappropriate pausing during long distance transport, while also enabling targeted delivery of cargo to highly exocytic, dynamic actin populations, beneath the plasma membrane.

P103**Board Number: B208****Conserved Binding Partner of Vertebrate and Invertebrate Class III Myosins.**M. Raval¹, K. Mecklenburg², S. Freed³, O.A. Quintero⁴, J.E. O'Tousa^{2,3}, C.M. Yengo⁵;¹Pennstate College of Medicine, Hershey, PA, ²Indiana University, South Bend, IN, ³Biology, University of Notre Dame, Notre Dame, IN, ⁴Department of Biology, University of Richmond, Richmond, VA,⁵Pennsylvania State Univ Coll Med, Hershey, PA

Class III myosins (MYO3A and MYO3B) are actin-based myosin motors which are proposed to function as transporters in actin rich structures such as the stereocilia of inner ear hair cells and calycal processes of photoreceptors (PRs). The first member of the MYO3 family was identified in *Drosophila* photoreceptors and called NINAC (Neither inactivation nor afterpotential C) based on its role in phototransduction. It was observed that NINAC null mutant flies undergo light and age dependent retinal degeneration. Previous work demonstrated that NINAC is found in a complex with a protein called retinophilin (RTP). In our current work we demonstrate that NINAC interacts with RTP via the proximal region of the tail domain, a region conserved in invertebrates. We also show that NINAC localization in the rhabdomere requires the presence of RTP. We find that the vertebrate homologue of RTP, membrane occupation nexus repeat protein (MORN4), interacts with MYO3A. We demonstrate that MORN4 gets transported to the filopodial tips of Cos7 cells in a MYO3A dependent manner. The presence of MORN4 enhances the MYO3A-induced elongation of filopodia. The domain structure of the MYO3A tail is encoded by 6 exons (30-35). Using tail exon deletion constructs we demonstrate that MYO3A tail exons 30-31 are the probable sites for MORN4 interaction. We also demonstrate that MYO3B which has a shorter tail (and lacks sequence similar to exon 30-31 of MYO3A tail) is unable to bind MORN4. We used expressed and purified GST labelled MORN4 to demonstrate that MORN4 specifically interacts with the MYO3A tail domain in GST pull down assays. The MYO3A tail domain exon 30 contains a proposed calmodulin (CaM)-binding IQ motif (IQ3) of unknown function. Thus, we hypothesized that Ca²⁺ may play a regulatory role in the MYO3A-MORN4 interaction. We observed a 30% increase in MORN4 tip localization in ionomycin treated Cos7 cells which were co-transfected with GFP-MYO3AΔK and mCherry-MORN4 as compared to control conditions. No significant change was observed in the MYO3A tip localization. Overall, our results suggest that the Retinophilin/NINAC interaction is conserved in vertebrate orthologs MORN4/MYO3A. In vertebrates we propose that MORN4 stabilizes MYO3A by tethering it to the membrane, thus allowing MYO3A to generate membrane tension and contribute to the process of elongation of actin rich protrusions.

P104**Board Number: B209****The TAP-MYO5A mouse: a Tandem Affinity Purification tag knockin mouse for the isolation and identification of proteins that interact with myosin Va.**C.J. Alexander*¹, W. Wagner*², N.A. Copeland³, N.A. Jenkins³, J.A. Hammer⁴;¹NHLBI, NIH, Bethesda, MD, ²ZMNH/UKE, Hamburg, Germany, ³The Methodist Hospital Research Institute, Houston, TX, ⁴LCB, NIH/NHLBI, Bethesda, MD

Myosin Va, a processive class V motor, is involved in the transport of diverse cargos, including melanosomes, endoplasmic reticulum (ER), and mRNAs. The creation of tools to identify proteins that interact with myosin Va should increase our understanding of the cellular processes supported by this myosin. Towards that end, we report here the generation of a tandem affinity purification (TAP) tag knockin mouse at the *MYO5A* locus. A recombineering-based approach was used to insert via homologous recombination a TAP-tag composed of the IgG binding domain of Protein A, a TEV cleavage site, and the FLAG epitope tag into *MYO5A* locus immediately after the initiation codon. Mice homozygous for the knockin allele, which express the TAP-tagged version of myosin Va (TAP-MyoVa) exclusively and under the control of the endogenous *MYO5A* promoter, exhibit normal coat color and no evidence of ataxia, arguing that TAP-MyoVa functions normally. Consistently, the dendritic spines of Purkinje neurons isolated from this mouse are fully loaded with ER, in contrast to the spines of Purkinje neurons from dilute (myosin Va null) mice, which are devoid of ER. Similarly, melanosomes are distributed normally in melanocytes from the TAP-MyoVa mouse, in contrast to melanocytes from dilute mice, where the organelles are concentrated in the cell center. Moreover, introduction of a CMV promoter-driven TAP-MyoVa construct into dilute melanocytes rescues melanosome distribution. Given this clear evidence that TAP-MyoVa is fully functional, we purified TAP-MyoVa and associated proteins directly from juvenile mouse cerebella excised from TAP-tagged mice and subjected the samples to mass spectroscopic analyses. Importantly, elutes contained several known myosin Va binding partners (Dynein light chain 1 and Neurofilament light chain), further verifying that TAP-MyoVa is fully functional. Moreover, we found numerous novel interacting proteins, including Annexin A2 and Elongation Factor 1A. The mouse model created here should facilitate the identification of novel myosin Va binding partners, which in turn should advance our understanding of the roles played by this myosin *in vivo*. *Authors contributed equally to this study

P105**Board Number: B210****Filopod Extension in Dictyostelium Requires MyTH4-FERM Myosin Motor Activity.**K.J. Petersen¹, G.G. Luxton², M.A. Titus²;¹Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, MN, ²Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN

Filopodia are cellular protrusions composed of bundled parallel actin filaments that initiate from the cortex with roles in cell adhesion and signaling during directed cell migration. MyTH4-FERM myosins (including Myosin 7, 10 and 15) localize to the distal tips of filopodia, stereocilia, and microvilli. Myo10 is required for filopod growth in mammalian cells while Myo7 is essential for filopod growth in Dictyostelium. We performed live cell imaging using GFP-tagged Myo7 in null cells. Myo7 rescued filopod formation and localized to filopodia tips and actin-based pseudopods. In pseudopods, enrichment at the leading edge near the cell membrane preceded the growth of filopodia. Tip extension velocity remained constant throughout the growing phase independent of Myo7 accumulation at the tip. A construct lacking MyTH4-FERM domains but including the putative SAH domain (Myo7 Motor) failed to rescue filopodia and did not co-localize with cortical actin. Neither of the two FERM domains were essential for rescue of filopod formation. Deletion of FERM2 led to increased production of substrate-adhered filopodia suggesting FERM2 might negatively regulate Myo7 activity in vivo. We tested this by identifying conserved residues implicated in auto-inhibition of Myo10 and expressing a mutated Myo7 (KK2333,2336AA) that also showed increased production of filopodia relative to wild type Myo7. A mutant lacking the motor domain (Myo7 Tail) was enriched near the cell membrane but failed to rescue filopod formation. Neither FERM domain was essential for localization, although a mutant lacking both domains failed to localize. These results suggest that normal formation of filopodia requires membrane-localized Myo7 with precisely regulated motor activity. (This work is supported by an NSF grant to MAT)

P106**Board Number: B211****Myosin X Reveals Dynamics and Function of Basal Filopodia.**D. Courson¹, K. Liu¹, A. Fanning¹, R. Cheney¹;¹Cell Biology and Physiology, University of North Carolina, Chapel Hill, NC

Though often depicted as simple and flat, the basal surface of monolayers of polarized epithelial cells is actually seething with protrusive activity. Using live-cell, time-lapse TIRF microscopy we visualize the dynamics of the basal surface of stable and migrating mammalian epithelial monolayers. Previous work showed that epithelial cells in migrating sheets extend cryptic lamellipodia, in the direction of migration. In this report we show that polarized mammalian epithelial cells (MDCK and Caco2) also have filopodia on their basal surface. Basal filopodia are numerous during monolayer formation and maturation, and probe ahead of the lamellipodia during migration.

Basal filopodia have gone largely unnoticed, however we demonstrate that myosin X (Myo10) marks the tips of epithelial filopodia of cells in isolation and basal filopodia of cells in polarized sheets. In wound healing assays Myo10 puncta are planar polarized at the leading edge of cells found throughout the monolayer, from deep within the sheet out to the leading edge. Basal filopodia and Myo10 puncta become less prominent as the monolayer becomes quiescent but reappear within minutes of wounding.

Myo10 and filopodia have both recently been shown to play roles in cancer metastasis. Our findings demonstrate that Myo10 and basal filopodia both directly affect cellular motility. We show that knocking down Myo10 results in the loss of basal filopodia and ~40% reduction in the rate of collective migration. A similar decrease in motility is observed by inhibition of filopodia via low dose cytochalasin. Interestingly, the difference in motility between wild type and Myo10 knockdown cells is lost upon treatment with low dose cytochalasin, consistent with the hypothesis that the loss of filopodia, rather than some other Myo10 function, is responsible for the observed motility defect in the knockdown cells. Ongoing work is examining the mechanism behind Myo10 recruitment, filopodia formation, and their roles in collective migration.

P107

Board Number: B212

Myosin 18A Co-assembles with Myosin II to Drive the Functional Diversity of Myosin II Bipolar Filaments.

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Bipolar filaments of nonmuscle myosin II (NMII) power myriad cellular and developmental processes. Here we provide evidence that myosin 18A α (M18A α) and myosin 18A β (M18A β), two M18A splice variants, co-assemble with NMII, opening the door to novel mechanisms of bipolar filament regulation. Structurally, M18A α and M18A β resemble NMII with extra domains at their N- and C-termini. Specifically, both isoforms possess a non-helical tailpiece that contains interaction sites for SH3 and PDZ domain-containing proteins, while M18A α also possesses a ~300 residue N-terminal extension containing a KE-rich region, a nucleotide-insensitive actin-binding site, and a PDZ domain. Importantly, both isoforms contain numerous substitutions in their active site and lack actin-activated ATPase activity, arguing that they are not motor proteins (Guzik-Lendrum et al., JBC, 2013). Using a combination of TIRF and structured-illumination microscopy (TIRF-SIM), which we used recently to show that NMIIA, IIB and IIC co-assemble into heterotypic bipolar filaments in live cells (Beach et al., Current Biology, 2014), we find clear evidence that M18A α and M18A β (both expressed and endogenous molecules) co-assemble with NMII in live cells in a variety of structures, including sub-nuclear stress fibers (SNSFs). Consistently, sedimentation, EM and single-molecule imaging show that purified M18A co-assembles with NMII *in vitro*. Incorporation of M18A into NMII bipolar filaments could serve to regulate filament assembly/disassembly. Indeed, excess M18A disrupts NMII filament assembly both *in vitro* and in live

cells. M18A levels *in vivo* are, however, markedly sub-stoichiometric to NMII (ranging from ~1:10 to ~1:100), and preliminary FRAP studies do not show an obvious effect of M18A depletion on NMII filament turnover. These results favor the idea that small numbers of M18A molecules dope NMII filaments to serve, via their extra N- and C-terminal domains, as adaptors to link the filament to cellular structures. Consistently, the targeting of M18A α to SNSFs depends on its PDZ domain, suggesting that this domain links SNSFs to the nuclear envelope. M18A could also serve to recruit molecules to the NMII filament. Consistent with this idea, we show M18A's non-helical tailpiece binds β PPIX, a Rac GEF with roles in adhesion and protrusion, via β PPIX's SH3 domain. Finally, we identify two additional M18A splice variants (M18A δ and M18A γ) that possess unique N- and C-terminal domains. Collectively, our data argue that diversity of NMII function in higher eukaryotes is driven in part by co-assembly with various spliced isoforms of M18A, which serve as adaptors to link the filaments to different cellular structures and signaling molecules without interfering with NMII motor activity.

P108

Board Number: B213

Myosin 18A localizes with myosin II at cell: cell junctions in epithelial cells and tissues.

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Class 18A myosins (M18A) are a poorly understood subclass of myosin with a domain architecture similar to that of myosin II (MII). Specifically, both M18A α and M18A β , two M18A isoforms generated by alternative splicing, consist of a "motor" domain followed by a short neck region, an extended coiled-coil domain, and a C-terminal non-helical tailpiece harboring binding sites for SH3 and PDZ domain-containing proteins. Myosin 18A α also possesses an N-terminal extension containing a KE-rich region, an ATP-insensitive actin-binding site, and a PDZ domain. Knockout of myosin 18A results in embryonic lethality in both mice and flies, suggesting a fundamental role in development. Despite their structural similarity to MII, M18A isoforms have no actin-activated ATPase activity and do not translocate actin filaments *in vitro*, suggesting that their functions do not require motor activity. Importantly, M18A and MII co-assemble via their extended coiled-coil domains into individual mixed bipolar filaments both *in vitro* and *in vivo*. This critical finding suggests that myosin 18A may serve to regulate MII filament turnover and/or act as an adaptor to link the filaments to different cellular structures/signaling molecules via its extra N- and C-terminal domains, all without interfering with MII motor activity. M18A is ubiquitously expressed across mammalian tissues, with elevated expression and isoform-specific expression in numerous cell types, including epithelia. In this study we determined the subcellular localization of M18A in polarized MDCK cell sheets and in cryo-sections of various mouse epithelia using a M18A-specific antibody. We find that M18A is concentrated at cell-cell junctions at the apical surface of polarized MDCK cells, a site where MII is known to be critical for maintaining the integrity of adherens junctions. Similarly, M18A is enriched in kidney proximal tubules and in intestinal brush border microvilli

specifically at cell: cell junctions where MII is also enriched. Finally, we find in secretory tissues such as the pancreas and salivary gland that M18A localizes along with MII onto the secretory granules after their fusion with the plasma membrane. Functional studies are underway to determine if M18A, like MII (Masedunskas et al, PNAS 2011), is required to facilitate the integration of the granular membranes into the apical plasma membrane, thereby completing the exocytic process. Together, our data suggest that M18A may be working together with MII to maintain the integrity of epithelia and to drive secretion in specialized tissues such as the pancreas and salivary gland.

P109

Board Number: B214

MYO19 ensures symmetric partitioning of mitochondria and coupling of mitochondrial segregation to cell division.

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During animal cell division, an actin-based ring cleaves the entire cell into two. Defects in this process can lead to chromosome mis-segregation, a hallmark of cancer, as well as to defects in cytoplasmic inheritance and the partitioning of organelles. Although a great deal is known about how chromosome segregation is coupled to the process of cell division, the way organelles coordinate their inheritance during partitioning to daughter cells is less well understood. Here, using a high-content live-imaging siRNA screen, we identify myosin-XIX (MYO19) as a novel regulator of cell division. Previously this actin-based motor was shown to control the interphase movement of mitochondria. Using live-cell imaging, our analysis shows that MYO19 is indeed localised to mitochondria, and that its silencing leads to asymmetric segregation of mitochondria during anaphase and telophase, as well as to defects in mitochondrial partitioning at cytokinesis. Moreover, MYO19 RNAi cells frequently fail to complete division. This cell division failure phenotype persists in cell lines stably expressing shRNA against MYO19, as these cell lines display a higher percentage of multinucleate cells, compared to controls. This phenotype appears to be due to the physical interference of cytokinesis machinery by mitochondria, as the phenotype can be mimicked using a treatment that blocks mitochondrial fission and rescued by decreasing mitochondrial fusion. The phenotype can also be rescued by exogenous expression of GFP-MYO19 refractory to RNAi treatment. Taken together, these data support a model whereby the actin-based myosin motor, MYO19, is responsible for the faithful segregation of mitochondria during cell division, and highlights the importance of coupling organelle inheritance to cytokinesis.

P110**Board Number: B215****Myosin XIX (Myo19) regulates actin-based mitochondrial dynamics.**X. Hu¹;¹Department of biology, Institute of Molecular Cell Biology, Münster, Germany

Mitochondria are highly mobile and dynamic organelles. They are involved in several important cellular functions, including energy production, metabolism, calcium buffering and apoptosis. Defects in their localization and dynamics lead to human disease. Myosin-XIX (Myo19) is a novel myosin in vertebrates that has been reported to function as an actin-based motor that associates with mitochondria. Myosins generally consist of a motor, neck and tail region. The tail region of Myo19 is necessary and sufficient for mitochondrial localization. However, at present it is not known how Myo19 associates with mitochondria and how it affects mitochondrial dynamics. We found that overexpression in HeLa cells of either the full length Myo19 or the Myo19 tail region resulted in dramatic alterations in mitochondria distribution, morphology and movement. Overexpression of Myo19 typically resulted in a collapse of mitochondria around the nucleus. Live cell imaging revealed that overexpression of Myo 19 induced in many continuously moving mitochondria a tadpole-like shape. Overexpression of the tail region of Myo19 caused a similar collapse of the mitochondria to the perinuclear region and the movement of individual mitochondria was hard to discern. The transport of mitochondria in dendrites and axons of neurons is particularly vital to meet local cellular energy demands and for buffering intracellular calcium. We found that mitochondrial length was significantly decreased in Myo19 overexpressing hippocampal neurons. No other differences were found between neurons transfected with RFP-Mito alone or RFP-Mito together with EGFP-Myo19 or EGFP-Myo19 tail regarding mitochondria density, velocity, run length, directionality etc. These results suggest that Myo19 is involved in regulating mitochondria morphology in neurons, but not transport. In conclusion, Myo19 regulates actin-based mitochondrial dynamics.

P111**Board Number: B216****Positively Charged Residues are Essential for Proper Localization of the MYO19 MyMOMA Domain to the Mitochondrial Outer Membrane.**P.R. Mehta¹, P.P. Singh¹, J.L. Hawthorne¹, N.Q. Wong¹, O.A. Quintero¹;¹Department of Biology, University of Richmond, Richmond, VA

Myosins are well characterized for their roles as molecular motors that convert the chemical energy of ATP hydrolysis into the mechanical force essential for cellular processes such as muscle contraction, cytokinesis, and intracellular transport. Class XIX myosin (MYO19) is an unconventional myosin found in organisms ranging from unicellular holozoans to mammals that contains a unique tail domain responsible for its interaction with mitochondria. The **Myo19 mitochondria outer membrane association domain (MyMOMA)** contains approximately 150 amino acids with a pI ~ 9, and is sufficient for

localization to the mitochondrial outer membrane. However, the minimal sequence required for mitochondrial binding and the specific amino acid residues required for mitochondrial targeting have not been identified. Here we report that a stretch of approximately 83 residues in the human MyMOMA domain is sufficient for mitochondrial outer membrane localization, and that two positively charged residues are essential for recognition of the mitochondrial outer membrane. We generated a suite of MyMOMA truncations based on sequence analysis of 13 vertebrate MYO19 orthologs, establishing the boundaries for truncations based on lack of sequence homology. The 83-amino acid minimal binding region is enriched for positively charged amino acids has a $pI \sim 10.5$. Endogenous MYO19 copurifies with mitochondria-enriched fractions via differential centrifugation, and can be released from those fractions by incubation in high pH buffer, suggesting that positively-charged residues may be involved in the MYO19 binding mechanism. We sequentially replaced positively charged residues in the minimal binding domain with uncharged residues via mutagenesis, identifying the specific residues required for interactions with the mitochondrial outer membrane, RK872-873. Interestingly, constructs containing the RK872-873AA mutation localized to the endoplasmic reticulum. To determine if ER-bound mutant MYO19 tail and mitochondria-bound wild type MYO19 tail display differences in strength of membrane interaction, we performed kinetic studies using permeabilization activated reduction in fluorescence (PARF) analysis. These studies indicated that both constructs display similar apparent off-rates. Taken together these data support the hypothesis that the MyMOMA domain contains a membrane-binding activity, and that membrane targeting is mediated through a yet-to-be determined mechanism requiring amino acids RK872-873.

P112

Board Number: B217

Characterizing the proximal protein interactions of Myosin-XIX (MYO19) via promiscuous biotin ligase.

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Myosin XIX (MYO19) is a motor protein that binds to mitochondria, and plays a role in the organization and dynamics of mitochondria. MYO19 consists of a motor domain, a 3 IQ motif-containing neck region, and a short tail. In conjunction with filamentous actin, the motor converts the energy released by ATP hydrolysis into useful force that is transmitted through the neck region to act upon the cargo-binding tail domain, thereby imparting force on the cargo. According to this model, there are multiple instances where interaction with other proteins is likely for MYO19 function, separate from the interaction with actin. We aim to elucidate the protein interactions of MYO19 by identifying proximal components and potential binding partners. To identify such interactors, we have created fusion constructs capable of tagging nearby proteins. By tagging MYO19 truncations with the promiscuous *E. coli* biotin ligase, BirA*/BioID, we are able to induce biotinylation of proximal cellular proteins. We hypothesize that the IQ motifs in the neck region bind EF-hand regulatory proteins such as calmodulin, and that the tail

domain interacts with proteins on the mitochondrial outer membrane. We first transfected HeLa cells with the BioID-MYO19 constructs, and incubated the cells in media containing biotin. We were able to observe expression of the MYO19/BioID constructs via fluorescence immunohistochemistry. Biotinylation was observed via immunostaining and anti-streptavidin blotting of HeLa cell lysates. Biotinylated proteins were isolated from lysates using magnetic strepavidin-coated beads, and the bound proteins processed via mass spectrometry. Through the analysis of these MYO19 associated proteins, we hope to better understand the cellular function of MYO19 by identifying proteins with which it may interact.

P113

Board Number: B218

Investigating the role of myosin 7b in intramicrovillar transport.

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The intestinal brush border serves as the sole site of nutrient absorption within the body and also acts as an important barrier against luminal pathogens. The brush border is comprised of membrane protrusions called microvilli that are found on the apical surface of enterocytes. These protrusions are supported by a core bundle of 20 to 30 parallel actin filaments with the plus ends oriented towards the lumen. Recently, our lab showed that two protocadherins, protocadherin-24 and mucin-like protocadherin, play a key role in the organization of the brush border during its assembly. These protocadherins interact to form a *trans*-heterophilic adhesion complex that physically connects the distal tips of microvilli and regulates the tight packing of the brush border. However, the mechanism of how these complexes are targeted to the tips of microvilli remains unknown. The microvillar protocadherins interact with two cytoplasmic binding partners, harmonin, a scaffolding protein, and myosin 7b (*myo7b*), an unconventional myosin. We hypothesize that the motor protein *myo7b* is responsible for transporting these complexes to the plus end of microvillar actin bundles. To test this hypothesis, we generated an shRNA-mediated knockdown of *myo7b* in CACO-2_{BBE} intestinal epithelial cells. Knockdown of *myo7b* results in reduced distal tip localization of the intermicrovillar adhesion complex (IMAC) components, as well as perturbations in microvillar clustering and brush border morphology. To further characterize the potential of *myo7b* as a transporter, we created a *myo7b* stable cell line using brush border-expressing LLC-PK1-CL4 kidney epithelial cells. Robust targeting of *myo7b* to the tips of microvilli occurs after several days of polarization. This localization is dependent on the tail domain as deletion of this region results in a loss of distal tip targeting. Previous studies of other myosins have indicated that cargo binding to the tail domain may promote motor processivity by inducing dimerization. To explore this possibility for *myo7b*, we engineered forced dimer constructs of the motor domain (MD) and full length *myo7b* using a GCN4 leucine zipper motif. The MD forced dimer targets in fully polarized LLC-PK1-CL4 cells, indicating that dimerization is sufficient for targeting and likely mediated by the tail domain through cargo binding. Additionally, we also examined how point mutations that disrupt motor domain function impact the distal tip targeting of *myo7b*. These data

suggest that active cycling and force generation are required for microvillar tip targeting. Taken together, these data indicate a role for myo7b in brush border formation through the localization of the IMAC to the tips of microvilli.

P114

Board Number: B219

Computational and experimental FRAP analyses of myosin XI-dependent vesicular transport show coupling with F-actin in polarized cell growth.

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Plant polarized cell growth is driven by the trafficking of secretory vesicles to the site of cellular expansion. Although the actin cytoskeleton and its associated motor myosin XI are heavily implicated in this process, little is known about their relative dynamics and binding properties *in vivo*. Here we applied fluorescent recovery after photo-bleaching (FRAP) techniques to the tip-growing cells of the moss *Physcomitrella patens* to investigate myosin XI dynamics and its relation to secretory vesicles. To complement our FRAP experiments, a three-dimensional Brownian motion simulation of FRAP was used to gain further insight into the dynamic behavior of myosin XI and vesicles. Our results clearly indicate that the dynamics of myosin XI at the cell apex differ significantly from those measured at the sub-apical region. Specifically, the rate of fluorescent recovery was found to be more rapid at the sub-apical region, indicating that a fraction of myosin XI is less mobile at the tip. To evaluate if the reduction on motility is dependent on F-actin, we depolymerized the actin using latrunculin B. This treatment significantly increased the mobile fraction of myosin XI at the cell apex, but not to the levels of the sub-apical region. To clarify this we used computer simulations that indicate that this discrepancy can be fully explained by cell boundary effects on the fluorescent recovery. In addition, our computer simulations have allowed us to estimate diffusion coefficients for myosin XI and vesicles, and at the same time estimate the fraction of myosin XI molecules associated with vesicles at the tip of the cell. Taken together our experimental results and simulation data support a model where myosin XI, secretory vesicles, and F-actin act cooperatively to drive polarized secretion and growth.

P115**Board Number: B220****Biochemical characterization of the interaction between Rab proteins and myosin XI in the moss *P. patens*.**S. Kaptur¹, J. Garbarino¹, M.L. Dubuke², F. Furt¹, E. Armstrong¹, M. Munson², L. Vidali¹;¹Biology/ Biotechnology, Worcester Polytechnic Institute, Worcester, MA, ²Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA

Actin-mediated trafficking plays an essential role in intracellular transport in all studied eukaryotic cells, including the moss, *Physcomitrella patens*. *P. patens* is an excellent model organism for studying actin-mediated trafficking because of its genetic tractability and simple cytology. Actin-mediated trafficking in *P. patens* is essential for polarized growth of its tip cells. The actin-associated motor, myosin XI, has been identified as an important protein for this process. Myosin XI is most similar to the class V myosins from yeast and animal cells. Similar to myosin V, *P. patens* myosin XI functions as a dimer with a head, neck, coiled-coil, and cargo binding domains. Myosin XI is hypothesized to function as a molecular motor carrying exocytotic vesicles along actin filaments to the plasma membrane. In mammalian and yeast cells, the association between myosin and its vesicle cargo is mediated by Rab-GTPases, such as Rab11 and Ypt31/32 respectively. We hypothesize that, in plants, a similar Rab-myosin interaction is necessary for vesicle transport. To evaluate this, we selected three classes of plant Rab proteins based on homology to Rab11 and Ypt31/32 and we are developing an in vitro binding assay to determine if these candidate Rab proteins have myosin XI binding activity.

Previous work in our laboratory also identified mutations in the myosin XI cargo-binding domain that failed to complement the RNAi-based knockdown of myosin XI. In this study, we aim to determine the structural consequences of the most severe mutations. We used PCR mutagenesis to generate protein expression constructs for three mutants of the myosin XI cargo-binding domain. We are purifying the wild type and mutant proteins, and plan to compare their folding and stability by circular dichroism, thermal denaturation, and potential for aggregation. Finally, using the Rab proteins identified as potential binding partners, we will determine if the loss-of-complementation in the RNAi screen was due to a binding defect between myosin XI and its cognate Rab. In this study, we use biochemical methods to determine the cause of the failure-to-complement of the myosin XI mutants and to determine if a subclass of Rab proteins is important for myosin XI binding to vesicles in plant cells.

Tubulins and Associated Proteins

P116

Board Number: B222

Effect of Tyrosine 18 Phosphorylation on the Conformation and Dynamics of Tau.

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Tau is a neuronal microtubule (MT) associated protein that performs numerous functions including stabilizing MTs, participating in signaling cascades and directly inhibiting kinesin-1 motility *in vitro*, but the relationship between Tau structure and function has yet to be fully elucidated. Recent single molecule experiments have shown that in addition to forming a conventional static complex, Tau can diffuse in an ATP-independent manner while bound to MTs, and the equilibrium between these populations differs with both Tau isoform and MT lattice structure. Furthermore, Tau can adopt a dynamic folded conformation in solution, stabilized through interactions between the N- and C-terminal regions of the molecule. In light of these findings, we hypothesize that the folded conformation of Tau represents the diffusive state and that the equilibrium between the diffusive and static states is mediated by post translational modifications that affect the stabilizing interactions of the N- and C-termini. We further hypothesize that phosphorylation at tyrosine 18 (Y18) in Tau's N-terminus helps stabilize the folded conformation and promotes the diffusive behavior of Tau on the MT surface. Correspondingly, dephosphorylation of Y18 leads to a shift from the diffusive to the static state on the MT surface. To test these hypotheses, phosphomimetic and unphosphorylatable control constructs of 3RS-Tau were made by substituting glutamic acid and alanine, respectively, at and around Y18. TIRF microscopy was used to examine the dynamic behavior of these Tau constructs on the MT surface, as well as their ability to inhibit kinesin motility.

P117

Board Number: B223

Down-regulation of stathmin is required for the phenotypic changes and classical activation of macrophages.

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Macrophages are major effector cells of the innate immune response with specialized capacity for recognition and elimination of pathogens, and present antigens to lymphocytes for adaptive immunity. Macrophages become activated upon exposure to pro-inflammatory cytokines and pathogenic stimuli.

Classical activation of macrophages with interferon- γ (IFN γ) and lipopolysaccharide (LPS) triggers a wide range of protein signalling events and morphological changes to induce the immune response. Our previous microtubule (MT) proteomic work revealed that stathmin association with MTs is considerably reduced in activated macrophages, which exhibit significantly enriched stabilized MTs. Here we show that there is a global decrease in the MT catastrophe protein stathmin level in activated macrophages using both immunoblotting and immunofluorescent microscopy. Our results suggested this is an LPS-specific response that induces proteasome-mediated degradation of stathmin. Furthermore, we explored the functions of stathmin down-regulation in activated macrophages by generating of a stable cell line overexpressing stathmin-GFP. We show that stathmin-GFP overexpression impacts cytoskeletal stability by reducing MT stabilization and acetylation, which significantly diminishes morphological changes associated with cell activation. In addition, we found that stathmin overexpression severely impaired immune functions such as complement receptor 3 (CR3) - mediated particle binding and macrophage activation. Overall, our results implicating a pivotal inhibitory role for stathmin in classically activated macrophages.

P118

Board Number: B224

Doublecortin bends growing microtubules.

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Microtubules are the stiffest members of the eukaryotic cytoskeleton. Despite their high persistence length, microtubules are highly bent in many cellular contexts, such as the neuronal growth cone. Here we show that Doublecortin (DCX), a microtubule-associated protein (MAP) expressed in developing neurons, bends growing microtubules in vitro. We measured the curvature, k , of microtubules bent by DCX using B-splines and show that DCX induces a characteristic bend in growing microtubules, whose curvature depends on DCX concentration. Our results demonstrate for the first time, that a MAP can directly modulate microtubule curvature.

P119

Board Number: B225

CP190-microtubule association is mediated by a novel N-terminal region that requires dimerization mediated by a flanking BTB domain.

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CP190 is a large, multi-domain protein, first identified as a centrosome protein with striking oscillatory localization over the course of the cell cycle. During interphase it has a well-established role within the

nucleus as a chromatin insulator. Upon nuclear envelope breakdown, there is a redistribution of CP190 to centrosomes and the mitotic spindle, with a population remaining associated with chromosomes. Previous work described a central CP190 region important for centrosome localization and microtubule (MT) binding. However, a detailed analysis of the CP190 N-terminal region has not been fully described. Here, we investigate CP190 in detail by performing domain analysis in cultured *Drosophila* S2 cells combined with protein structure determination by x-ray crystallography and *in vitro* biochemical characterization.

Our analysis of CP190 identified a novel centrosome targeting and microtubule (MT) interacting region. This previously uncharacterized CP190 N-terminal region robustly localizes to interphase MTs and is enriched at the growing MT plus ends. This region consists of a highly conserved BTB domain and a linker region that directly interacts with taxol-stabilized MTs *in vitro*. We present the 2.6 Å resolution structure of the CP190 N-terminal 126 amino acids, which adopts a canonical BTB domain fold and exists as a stable dimer in solution. The CP190 BTB domain is important for robust MT localization. The ability of the linker region to colocalize with MTs in cell culture requires BTB-domain mediated dimerization, though MT colocalization can be rescued via artificial dimerization, replacing the BTB domain with either GST or the GCN4 coiled-coil. These data suggest a possible regulatory role for the BTB domain in modulating CP190 association with the MT network. Finally, in live cells, we see that this N-terminal region and the artificially dimerized linker localize to the kinetochore.

P120

Board Number: B226

The Microtubule tip is a central node for amplification of calcium signals.

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Growing microtubule (MT) tips establish a transient interaction with endoplasmic reticulum (ER), the main Ca²⁺ store in the cell, but the functional outcome of these events are poorly understood. Here we demonstrate that growing MTs serve as the binding platform for the inositol 1,4,5-trisphosphate receptors (IP₃Rs), the Ca²⁺ gating channel on the ER membrane. End binding protein 3 (EB3), a core element of plus end tracking protein (+TIP) complex, interacts with the IP₃R channel and triggers its clustering. This interaction provides the molecular basis for spatio-temporal organization of Ca²⁺ signals and determines the overall outcome of the complex cellular response. Targeting EB3-IP₃R interactions in endothelial cells stabilizes Vascular Endothelial (VE)-cadherin adhesions and suppresses vascular inflammation suggesting that MT-dependent amplification of Ca²⁺ signaling is a significant pathological component that can be targeted therapeutically. These findings establish the primacy of the EB3-IP₃R complex as a central signaling node in vascular pathophysiology.

P121**Board Number: B227****An elastic shield coupled to rigid interface provides mechanical integrity to myonuclei.**S. Wang¹, T. Volk¹;¹Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel

Differ from most other cell types, striated muscle cells contain high number of nuclei distributed evenly on the surface of the myotube and are subjected to variable mechanical stress during muscle contraction.

The characteristic oval myonuclear shape and positioning have been linked to transcriptional regulation, as well as to correct export of mRNAs and proteins. Thus, correct nuclear shape and position are critical for proper muscle function. Consistently, numerous muscular dystrophies and (cardio-)myopathies exhibit nuclear mis-positioning and aggregation.

Previously, we revealed the essential role and molecular interaction between the two *Drosophila* KASH-domain proteins Klar and MSP-300/Nesprin in promoting the even distribution of myonuclei and their anchorage to the acto-myosin in striated muscle fibers. Recently, by performing muscle-specific RNAi screen, we identified additional proteins involved in the core mechanism for myonuclear shaping and distribution process. We demonstrate that the Spectraplakkin Shortstop (Shot) is a crucial stabilizing component of a microtubule (MT)-based network that surrounds the myonucleus. Moreover, Shot and MSP-300 interact genetically with each other and their stable attachment to the nuclear envelope requires their mutual existence.

Furthermore, both Shot and EB1 display a perinuclear localization pattern like MSP-300, and their knockdown leads to severe disruption of myonuclear shape, supporting a cooperative and reciprocal activity between MSP-300, Shot and EB1 in organizing the perinuclear MT network. Additionally, knockdown of Shot or EB1 perturbs nuclear shape and meanwhile induce ectopic perinuclear F-actin accumulation, indicating a potential involvement in the negative regulation of F-actin polymerization. Finally, our experiments demonstrate that MSP-300, Shot, and EB1 are essential to protect and maintain myonuclear architecture during mechanical stress implicated during muscle contraction.

Together, all these components form a perinuclear flexible shield essential for protecting myonuclei from intrinsic or extrinsic forces. The loss of this scaffold resulted in significantly aberrant nuclear morphology and a subsequent abnormal distribution of factors essential to maintain proper nuclear architecture. Overall, we propose a novel mechanism for protecting myonuclear morphology and reveal its critical link to correct distribution of nuclear factors in differentiated muscle fibres. These findings may shed light on the cause of various muscular dystrophies.

P122**Board Number: B228****Induced changes in the dynamics of EB1-mNeonGreen and microtubules in *Chlamydomonas*.**Y. Liu¹, K.F. Lehtreck², P. Yang¹;¹Department of Biological Sciences, Marquette University, Milwaukee, WI, ²Cellular Biology, University of Georgia, Athens, GA

EB1 is a master plus end tracker central to microtubule-based processes. It appears like comets near the plus end of only growing microtubules where GTP in tubulin is stochastically hydrolyzed to GDP. At this position EB1 could recruit effectors that modulate microtubule dynamics or harness cellular structures to plus ends. Yet EB1 is also localized outside plus ends, such as basal bodies and the centrosome. The molecular basis for the distinct locations remains to be resolved. To investigate the factors affecting EB1's locations, we express fluorescing EB1 as endogenous EB1 in *Chlamydomonas* that generate a few types of microtubules. Both EB1-GFP and EB1-mNeonGreen decorated basal bodies and microtubule plus ends at the flagellar tip. However, contrary to EB1-GFP that was entirely obscured by intense autofluorescence from photosynthesis pigments, EB1-mNeonGreen exhibited comet-like patterns typical of EB1 at the plus end of profuse growing microtubules in the cell body. Therefore like EB1-GFP, EB1-mNeonGreen could report the behaviors of EB1 and mNeonGreen is conducive for live imaging of photosynthetic cells. In some preparations - depending on media content, cell density and illumination for microscopy – the comets vanished, perhaps due to diminished growing microtubules; or, unexpectedly, EB1-mNeonGreen appeared like static fibers, some of which resembles the rootlets comprised with characteristic bundles of 2 or 4 microtubules. We are currently defining the conditions that induce changes in the dynamics and interplay of microtubules and EB1.

P123**Board Number: B229****The dynamics of EB1 in *Chlamydomonas* flagella.**Y. Liu¹, A. Harris², K.F. Lehtreck², P. Yang¹;¹Department of Biological Sciences, Marquette University, Milwaukee, WI, ²Cellular Biology, University of Georgia, Athens, GA

The motor-driven intraflagellar transport (IFT) has been considered as the principle trafficking modality for gated lengthy flagella except for small particles that rely on passive diffusion. Although EB1 is best known for binding to the plus end of growing microtubules, immunolocalization revealed EB1 at basal bodies near the entrance and the tip of *Chlamydomonas* flagella regardless growing, full length or shrinking. To reveal how EB1 molecules travel to and remain at the tip, we expressed EB1-GFP as endogenous EB1 in a few *Chlamydomonas* strains, including temperature sensitive IFT mutant *fla10*, in which anterograde IFT could be switched off at 32°C. In all strains and temperatures examined, EB1-GFP is present at the tip and basal bodies as well as the flagellar shaft, albeit dimmer. Fluorescence recovery

after photobleaching (FRAP) analysis showed that EB1-GFP moves directionally but far faster than IFT. The tip population in steady-state flagella fully recovers within 2-3 min post bleaching. Further analysis suggests that EB1 forms a stable pool at the flagellar tip which slowly exchanges with the population in the shaft. EB1-GFP is expected to form a dimer of about 120 kDa. Our data suggests that IFT-independent movement is sufficient to rapidly translocate cytoplasmic proteins of more than 100 kD in size to the flagellar tip. We are currently investigating the nature of IFT-independent directional rapid movement of EB1 and whether the non-canonical behavior of EB1 at the flagellar tip is linked to the dynamics of the axonemal microtubules.

P124

Board Number: B230

TIP150 regulation in cell migration and functional relevance interaction with the cytoskeleton.

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The regulation of cell migration is a highly complex process that is often compromised when cancer cells become metastatic, migrating from primary-to-secondary locations. The microtubule cytoskeleton is necessary for cell migration, but how microtubules and microtubule-associated proteins regulate multiple pathways promoting cell migration remains unclear. Microtubule plus-end binding proteins (+TIPs) are emerging as important players in many cellular functions, including cell migration. Here we identify a +TIP, TIP150, that promotes cell migration. TIP150 accumulates at growing microtubule plus ends through interaction with the EB1. The EB1-dependent +TIP activity of TIP150 is required for cell migration, as well as for interactions with both actin and microtubule. Here we describe the characterization and functional identification of TIP150 and its association in cellular migration. We hypothesize that TIP150 helps regulate cell migration and links actin to microtubules. To this end, we used a series of biochemical and biophotonic experiments to show TIP150 is necessary for directional cell migration and potentially links to microtubules and actin, further offering additional evidence of the importance of microtubule dynamics in the cell migration process.

P125**Board Number: B231****The role of +TIPs in the remodelling of microtubules during intestinal epithelial differentiation.**

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Microtubule dynamics and organisation play a key role in determining cellular function in differentiated epithelial cells. The intestine contains a hierarchy of epithelial development and provides an ideal model to study microtubule organisation. Stem cells located at the base of the crypt give rise to transit-amplifying cells that divide, differentiate and migrate up the crypt and into the villus in the small intestine or the surface epithelium in the colon.

Here we show that epithelial cells located at the bottom of intestinal crypts contain an umbrella-like microtubule array with minus-ends anchored at apical centrosomes. However, in the villus centrosomal anchorage is lost and a stabilised apico-basal microtubule array forms with minus-ends anchored at apical non-centrosomal sites. Interestingly, some of the microtubule plus-end tracking proteins (+TIPs) show distinct expression patterns in the intestine. For example, the end-binding protein EB2 is highly expressed at the bottom of crypts but downregulated in the transit amplifying and differentiated enterocytes. EB2 reduction in intestinal epithelial cells leads to microtubule stabilisation and crosslinking to actin filaments, suggesting that EB2 downregulation plays an important role in apico-basal array formation. EB1 is expressed throughout the intestine but a shift from comet only to microtubule lattice association is evident in differentiating cells with distinct apico-basal bundles.

Loss of microtubule centrosomal anchorage coincides with the redeployment of the centrosomal anchoring protein ninein to apical junctional sites. Interestingly, the +TIPs CLIP-170 and p150 are also found at these anchoring sites. Analysis of intestinal villus and surface epithelium of CLIP-170/115 knockout mice reveal a loss of ninein from these anchoring sites. Furthermore, functional inhibition in epithelial cell lines suggests that CLIP-170 along with active Rac1 and IQGAP1 ensures efficient redeployment of ninein by facilitating microtubule plus-end cortical capture at adherens junctions. Using recent advancements in intestinal organoid cultures we have further investigated the role of these +TIP proteins in epithelial differentiation. Our data suggest tight regulation of both expression and localisation of EBs and CLIP-170 during intestinal differentiation and show that these +TIPs are critical for microtubule reorganisation and epithelial differentiation.

P126**Board Number: B232****Microtubule +TIP protein EB1 binds to GTP and undergoes dissociation from dimer to monomers upon binding with GTP.**K. K. Gireesh¹, J. S. Sreeja¹, S. Chakraborti², P. Singh¹, G. E. Thomas¹, H. Gupta¹, T. Manna¹;¹School of Biology, Indian Institute of Science Education and Research Thiruvananthapuram, Kerala, India, ²Department of Neurobiology signalling and Cancer, Institut Curie, Orsay Cedex, France

Dynamic properties of microtubules predominantly rely on the conformational status of their growing plus end. The process of conformational arrangement is tightly regulated by a wide range of proteins localized at the ends. One of the highly abundant proteins localized at the plus end is EB1, which autonomously tracks the growing end and regulates the plus end dynamics. The mechanism through which EB1 recognizes the plus ends has been under intense scrutiny in recent years. Previous studies have indicated that EB1 can recognize the GTP-bound tubulin structures at the plus ends and it localizes on the microtubule surface at a site close to the exchangeable GTP-binding site of tubulin. Furthermore, its binding has been shown to promote maturation of the plus end and accelerate the GTP hydrolysis cycle at the end. Although the GTP-dependent structural change in tubulin has been demonstrated as a critical determinant for recognition of the plus ends by EB1, the effect of GTP on the structure of EB1 has remained unclear. In this study, we have used spectroscopic, calorimetric and biochemical methods to analyze the effect of GTP on EB1 *in vitro*. Isothermal titration calorimetry and tryptophan fluorescence quenching experiments demonstrated that EB1 binds to GTP with a dissociation constant $\sim 30 \mu\text{M}$. Circular dichroism measurements showed that EB1 undergoes changes in its secondary structure upon binding with GTP. Size exclusion chromatography and the urea-induced unfolding analyses revealed that GTP-binding induces dissociation of the EB1 dimer to its monomers. Size exclusion chromatography followed by biochemical analysis determined that EB1-GTP binding involves association of approximately one molecule of GTP per EB1 monomer. We also found that GTP did not affect the stability of the dimer formed by the C-terminus (191-268) of EB1. Detailed analysis further revealed that the GTP specifically binds to the N-terminus (1-130) of EB1 and induces conformational changes in the N-terminus, suggesting that the GTP-induced conformational change in the N-terminus destabilizes the monomer-monomer interaction in the full length EB1 dimer. The results reveal a hitherto unknown GTP-dependent mechanism of dimer to monomer transition in EB1 and further implicate its possible role in regulation of EB1 dimer vs. monomer stability and the plus end regulation in cells.

P127**Board Number: B233****Drosophila melanogaster Msps TOG3 utilizes unique structural elements to promote domain stability and maintain an integral tubulin-binding surface.**A.E. Howard^{1,2}, K.C. Slep³;¹Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC, ²Molecular and Cellular Biophysics Program, University of North Carolina, Chapel Hill, NC, ³Department of Biology, University of North Carolina, Chapel Hill, NC

XMAP215 family members are potent plus-end microtubule (MT) polymerases. Mutation or depletion of XMAP215 decreases MT polymerization rates and contributes to abnormal spindle phenotypes. The XMAP215 family utilizes an array of tumor overexpressed gene (TOG) domains, consisting of six (A-F) helix-turn-helix HEAT motifs, to bind tubulin. Recently, we showed that XMAP215 family TOG domains have differential, positionally conserved architectures across the array, highlighted by the similar architectures of TOG1 and TOG2, and the differential, comparative architecture of TOG4. Differential TOG domain architectures have the potential to contribute to unique TOG-tubulin interactions, with implications for position-dependent TOG domain tubulin-binding activities and function within the XMAP215 MT polymerization mechanism. Although structures of TOG domains 1, 2, and 4 are well-described, structural and mechanistic information characterizing TOG domains 3 and 5 are outstanding. Here we present the crystal structure of *Drosophila melanogaster* Msps TOG3 to 2.3 Å resolution. Msps TOG3 has two unique features; the first is a unique C-terminal tail that interlocks and stabilizes the ultimate four HEAT repeats, the second is a unique architecture in HEAT repeat B. The Msps TOG3 C-terminal tail has conserved determinants, including an arginine (R816), that form multiple contacts with HEAT repeats C-F. Mutational and circular dichroism studies demonstrate that the TOG3 C-terminal tail promotes TOG3 domain stability. Pairwise structural alignments of TOG3 with other TOG domain structures determined to date show that TOG3 is most similar to TOG domains 1 and 2, with rmsd values of 2.4 and 3.0 Å, respectively. In contrast, TOG3 diverges from the architecture of TOG4 with a rmsd of 4.6 Å. Docking TOG3 onto recently solved Stu2 TOG1- and TOG2-tubulin structures suggests that TOG3 uses similar, conserved tubulin-binding intra-HEAT loop residues to interact with tubulin. This suggests that TOG3 has maintained a TOG1- and TOG2-like TOG-tubulin binding mode despite structural divergence in the second HEAT repeat. The similarity of TOGs 1-3 and the divergence of TOG4 suggest that a polarized, differential TOG architecture may play a key mechanistic role in XMAP215-dependent MT polymerization activity.

P128**Board Number: B234****STU2/XMAP215 interacts with SUMO and the sumoylation machinery.**A. Alonso¹, M. Greenlee¹, R.K. Miller¹;¹Biochemistry and Molecular Biology, Oklahoma State University, Stillwater, OK

Sumoylation is a ubiquitin-like modification that regulates a wide variety of fundamental cellular processes, including DNA repair, transcription, and cell-cycle transitions. However, it is currently not known how sumoylation regulates microtubules. Microtubules perform important roles in the structure of the mitotic spindle as well as serving as tracks for transportation of cargo to various destinations in the cell. Sumoylation has only recently been shown to regulate spindle positioning. Our lab has previously shown that the LIS1 homologue Pac1 is modified *in vivo* by SUMO and ubiquitin. Pac1 is important for recruiting dynein to the plus end of the microtubule. Dynein is subsequently “off-loaded” to the cortex where it pulls on cytoplasmic microtubules to move the mitotic spindle across the bud neck, a key step in positioning the mitotic spindle. Using two-hybrid analysis, we show that PAC1 interacts with STU2. The C-terminus of Stu2 is sufficient for this interaction. Stu2 is the yeast homologue of XMAP215 and stabilizes microtubules by facilitating the loading of tubulin dimers onto microtubule ends. Stu2 can also de-stabilize microtubules by sterically interfering with tubulin addition. Here we show by two-hybrid analysis that Stu2 interacts with Smt3/SUMO. Stu2 also interacts with the E2 Ubc9, and the E3 Nfi1 of the sumoylation pathway. Stu2/XMAP215 also interacts with SUMO *in vitro* and *in vivo*. Using an *in vitro* sumoylation assay, four shifted bands of Stu2p can be observed. Using a temperature sensitive allele of the ubiquitin like protease, Ulp1, Stu2 displays a higher molecular weight band *in vivo* that cross reacts with SUMO by immunoblotting. In whole cell extracts, Pac1 shifts more extensively than Stu2 by western blotting. Considering that Stu2 is the eighth class of MAP shown to interact with SUMO, we suggest that sumoylation may be a general mechanism for regulating microtubule-associated proteins.

P129**Board Number: B235****Stu2, the budding yeast XMAP215/DIS1 homolog, is a microtubule polymerase with higher activity on yeast tubulin than on mammalian-brain tubulin.**M. Podolski¹, J. Howard¹;¹Molecular Biophysics and Biochemistry, Yale University, New Haven, CT

Stu2 is the budding yeast member of the XMAP215/Dis1 family of microtubule polymerases. It is essential in cell division, allowing proper spindle orientation and metaphase chromosome alignment, as well as spindle elongation during anaphase. Despite Stu2 having a phenotype that suggests it promotes microtubule growth, like the other members of XMAP215/Dis1 family, studies till now with purified Stu2 indicate only that it antagonizes microtubule growth. One potential explanation for these contradictory findings is that the assays were performed with mammalian brain tubulin, which may not be the right

substrate to test the activity of Stu2, given that yeast and brain tubulins are quite divergent in sequence, and that the vertebrate tubulins are subject to many post-translational modifications. To test this possibility, we reconstituted the activity of Stu2 with purified budding yeast tubulin. We found that Stu2 accelerates microtubule growth in yeast tubulin several fold, similar to the acceleration reported for XMAP215 in porcine brain tubulin. Furthermore, Stu2 accelerates polymerization in yeast tubulin to a much greater extent than it does in porcine brain tubulin, and the concentration of Stu2 required to reach 50% maximum activity in yeast tubulin was nearly two orders of magnitude lower than in porcine brain tubulin. We conclude that Stu2 is a microtubule polymerase, like its relatives, and that its activity is considerably higher in yeast tubulin compared to mammalian brain tubulin. These studies highlight significant differences between tubulins from different sources and the importance of using conspecific tubulin when studying microtubule associated proteins.

P130

Board Number: B236

Alcohol metabolism by Cytochrome P450 2E1 does not contribute to hepatocellular defects associated with tubulin acetylation, but does impair STAT5 nuclear translocation.

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Although alcoholic liver disease has been clinically well described, the molecular mechanisms that promote hepatotoxicity are not yet fully understood. The liver metabolizes alcohol using two enzymes, alcohol dehydrogenase (ADH) and cytochrome P450 2E1 (CYP 2E1). Both ADH and CYP 2E1 metabolize alcohol into acetaldehyde, but CYP 2E1 activity also results in the production of reactive oxygen species (ROS) that lead to oxidative stress. We have previously shown that microtubules are more highly acetylated in alcohol-treated polarized hepatic WIF-B cells and livers from alcohol fed rats. We further determined that alcohol metabolism to acetaldehyde by ADH is required for enhanced acetylation. However the role of CYP2E1-mediated metabolites and ROS in tubulin acetylation was not examined. To determine if CYP 2E1-mediated alcohol metabolism is required for enhanced acetylation, we treated cells with 100 μ M diallyl sulfide (DAS), a CYP 2E1 inhibitor, or 5 mM N-Acetyl Cysteine (NAC), an antioxidant that decreases ROS, in the presence of 50 mM alcohol. Alcohol-treated cells revealed that inhibition of CYP 2E1 with DAS did not alter enhanced tubulin acetylation, supporting that its activity does not mediate the effect. Consistent with this result is the finding that DAS treatment failed to rescue the impaired ASGP-R internalization observed in alcohol-treated cells. However, inhibition of CYP 2E1 did reverse the STAT5B nuclear translocation impairment that has been previously observed in alcohol-treated cells. It is believed that this is caused by a defect in STAT5B activation by Jak2 kinase, which is independent of the microtubule network, further supporting that CYP 2E1 alcohol metabolism does not affect microtubule hyperacetylation.

P131**Board Number: B237****Alcohol-induced tubulin acetylation sites identified by mass spectrometry.**J. Groebner¹, D. Tuma², P. Tuma¹;¹Department of Biology, Catholic Univ America, Washington, DC, ²Department of Internal Medicine, University of Nebraska, Omaha, NE

Although the progression of alcoholic liver disease is clinically well-described, the mechanisms leading to alcohol-induced liver damage remain elusive. Previously, we determined that microtubules are hyperacetylated and more stable in ethanol-treated WIF-B cells, liver slices and in livers from ethanol-fed rats. Our focus is to determine whether tubulin hyperacetylation can explain alcohol-induced defects in microtubule-dependent protein trafficking. Previously, we determined that transport of newly synthesized proteins from the Golgi to the basolateral surface and STAT5B nuclear translocation are impaired by alcohol metabolism. Recently, we confirmed that delivery of apical proteins from the basolateral-to-apical membrane via transcytosis is also impaired in ethanol-treated WIF-B cells. Unlike in control cells, transcytosing proteins accumulated sub-apically and aligned along acetylated microtubules in ethanol-treated cells. Both dynein and dynactin colocalized with transcytosing proteins, but only dynein (not dynactin), more tightly associated with microtubules from ethanol-treated cells. Thus, we conclude that enhanced dynein binding to microtubules in ethanol-treated cells leads to decreased motor processivity resulting in vesicle stalling and impaired delivery. A common feature of these delivery routes is regulation by microtubule-based motors. The “tubulin code” hypothesis proposes that microtubule-based motor trafficking for specific cellular functions may be regulated by tubulin isotype composition and post-translation modifications. To more directly examine microtubule hyperacetylation in alcohol-treated cells, we have analyzed purified taxol-stabilized microtubules from control, trichostatin A (TSA - leads to global protein acetylation)- and ethanol-treated WIF-B cells by mass spectrometry. In preliminary studies, we have successfully recovered both α - and β -tubulin with ~60% coverage in both control, TSA- and ethanol-treated cells. As predicted, Lys40 (the known α -tubulin acetylated site) was fully acetylated in ethanol-treated cells with all recovered fragments being acetylated. In contrast, no acetylated Lys40 containing peptides were recovered from control tubulin. We also identified novel acetylated lysines in the C-terminal half of α -tubulin (in ethanol-treated cells) and β -tubulin (in both control, TSA- and ethanol-treated cells). One site was more highly acetylated in ethanol-treated cells. We are currently generating site specific lysine mutants to directly identify which residues contribute to impaired motor properties and defects in protein trafficking.

P132**Board Number: B238** **α -Tubulin Acetyltransferase α TAT1 Preferentially Targets Tapered Microtubule Ends with Exposed Lys40 Acetylation Sites.**C.E. Coombes¹, A. Yamamoto¹, T. Reid¹, M. McClellan¹, G.G. Luxton², J. Alper³, M. Gardner¹;¹Genetics, Cell Biology, and Development, University of Minnesota, Minneapolis, MN, ²Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN, ³Molecular Biophysics and Biochemistry, Yale University, New Haven, CT

Microtubules are structural polymers inside of cells that are subject to post-translational modifications. These post-translational modifications help to create functionally distinct subsets of microtubule networks in the cell. Acetylation is unique from other post-translational modifications in that it is the only modification which takes place in the hollow lumen of the microtubule. While it is known that the α -tubulin acetyltransferase α TAT1 is responsible for the majority of the microtubule acetylation that is observed in cells, the mechanism for how α TAT1 accesses the Lys40 acetylation site inside of the microtubule lumen is not fully understood, and remains an important topic of investigation. To examine how α TAT1 accesses the luminal acetylation site, we performed a combination of biochemical assays, fluorescence and electron microscopy experiments, and simulations. We found that the microtubule α -tubulin acetylation rate is limited by accessibility of the enzyme to the lumen, and that α TAT1 preferentially targets tapered microtubule ends with exposed Lys40 acetylation sites. This preference for tapered microtubule ends may play a role in the specific targeting of α TAT1 to subsets of cellular microtubules. These results provide important insights into the mechanism for α TAT1 microtubule acetylation and its dependence on the microtubule structure.

P133**Board Number: B239****Mouse Sperm Associate Antigen 6 Regulates Cell Growth, Cell Migration And Ciliogenesis Through Activation of Acetylated Tubulin In Mouse Embryonic Fibroblast Cells.**Z. Zhang¹;¹Obstetrics/Gynecology, Virginia Commonwealth University, Richmond, VA

Mammalian Spag6 is the orthologue of Chlamydomonas PF16, which encodes a protein localized in the axoneme central apparatus of flagella and regulates flagellar/cilia motility. Most Spag6-deficient mice are smaller in size than their littermates, suggesting that mouse SPAG6 might also play roles other than regulating flagellar/cilia motility. When mouse embryonic fibroblasts (MEFs) were cultured from Spag6-deficient mice and their littermates, it was found that both primary and immortalized Spag6-deficient MEFs proliferated at a much slower rate than the wild-type MEFs of the same passages. Spag6-deficient MEFs also had enlarged cell size, and they appeared to be more flattened and had more cytoplasmic

vesicles than the wild-type MEFs. Transwell chambers and wound-healing assays demonstrated that the Spag6-deficient MEFs had dramatically reduced migration ability. During migration, polarized distribution of α -tubulin, a subunit of microtubule, and F-actin, another cytoskeleton component observed in the wild-type MEFs was never seen in the Spag6-deficient MEFs. Abnormally dividing cells were observed in the Spag6-deficient MEFs as demonstrated by multiple centrioles illustrated by γ -tubulin staining. Spag6-deficient MEFs also demonstrated ciliogenesis defects; the percentage of cells with primary cilia was significantly reduced compared to the wild-type MEFs, and some Spag6-deficient MEFs had multiple cilia. The expression level of acetylated tubulin, a marker for cilia, and also an indicator for microtubule stability, was dramatically reduced in the Spag6-deficient MEFs. In contrast, overexpressing SPAG6 in multiple cultured mammalian cells significantly increased acetylated tubulin expression level, and SPAG6 co-localized with acetylated tubulin. Our studies have revealed unexpected roles of mouse SPAG6; it not only regulates flagellar/cilia motility, it also regulates cell proliferation, cell migration, cell division, and ciliogenesis, perhaps through influencing acetylated tubulin expression and microtubule stability. Given F-actin distribution is also changed in the Spag6-deficient MEFs, SPAG6 might also influence functions of other cytoskeleton systems.

P134

Board Number: B240

Not just charged strings: binding of Tubulin's disordered tails to VDAC is regulated by small sequence changes and by posttranslational modification.

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The tubulin alpha-beta heterodimer is the subunit of microtubules (MT) but is also a freely diffusing cytoplasmic protein in its own right which can find binding partners distinct from those for MT. One of these partners is the mitochondrial outer membrane protein VDAC (Voltage Dependent Anion Channel), which mediates small molecule traffic into and out of the mitochondria. We previously showed that tubulin binding to VDAC blocks channel traffic and reduces oxidative metabolism, and that this requires the unstructured anionic tail peptides found on both the alpha- and beta-tubulin subunits. But those studies left unclear if the tubulin body per se is required, whether the alpha- and beta-tails contribute equally to VDAC blockade, and the role, if any, of tubulin posttranslational modifications (PTM), which mostly occur on the tails. These intrinsically disordered tails contain only ~3% of the mass but ~40% of the net charge on the tubulin heterodimer. Earlier studies showed that anionic polymers could cause VDAC blockade, but gave no indication of sequence sensitivity. Here we use single molecule binding studies to show that channel closure is due to the tails alone, since grafting them to albumin gives the same VDAC closure as tubulin. We show that the beta-tail is ~100-fold more potent than the alpha-tail, and that this difference is largely due to the presence or absence of a terminal tyrosine. The activation of the alpha-tail by detyrosination is reversed by subsequent removal of the next residue. We also show that nitration of tyrosine reverses the tyrosine inhibition of binding and even induces prolonged VDAC closures. Our results demonstrate that small changes in sequence or PTM of the disordered tails of

tubulin result in substantial changes in VDAC closure. We expect that this pattern may be repeated when comparing the numerous isotypes of alpha- and beta-tubulin, as well as other PTMs. These results also provide a sensitive system to study biology of disordered protein sequences, and their sensitivity to small chemical changes.

P135

Board Number: B241

Zeta-tubulin Orients Cilia in Multiciliated Epithelial Cells.

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Tubulins are a conserved superfamily of proteins that are important in the structure and function of the microtubule cytoskeleton. Members of the alpha-tubulin, beta-tubulin, and gamma-tubulin families are found in all eukaryotes, and are the structural elements of the microtubule polymer, and the main nucleator of that polymer, respectively. Members of the delta-tubulin and epsilon-tubulin families are present in many, but not all, eukaryotes and are associated with centrioles. We have characterized in vertebrates a member of another tubulin family, which most closely resembles tubulins previously named eta-tubulin or zeta-tubulin. Based on an assessment of the entire tubulin superfamily we will refer to this as the zeta-tubulin family. Zeta-tubulin is present in many single-celled eukaryotes, chordates, and marsupial mammals, but is absent in *Drosophila*, *C. elegans*, zebrafish, and placental mammals. Analysis of genome sequences indicates that zeta-tubulin is the last remaining tubulin family member to be characterized in vertebrates. Zeta-tubulin is the least well-conserved of the tubulin families, both in terms of the number of lineages that have lost the gene and in the divergence of the protein within branches that have retained it. We have characterized *Xenopus laevis* zeta-tubulin in a variety of cell lines and developmental stages. In the multiciliated skin cells of the frog embryo, zeta-tubulin is a basal body component, with a pattern of localization consistent with it being a part of the basal foot. Remarkably, *Xenopus* zeta-tubulin is able to localize to basal bodies in multiciliated cells of mouse, which has lost the gene for this tubulin. This suggests that the module of function, perhaps the basal foot, is conserved and that another tubulin family member might take the place of zeta-tubulin in species in which it is lost. Depletion of zeta-tubulin results in disorganization of basal body distribution and polarity in multiciliated cells, but no apparent phenotypes in other cell types. In contrast with multiciliated cells, zeta-tubulin in cycling cells does not localize to centrioles and instead is associated with the TRiC-CCT complex, a large cytoplasmic chaperone that is responsible for folding actin, tubulin, and other proteins. We conclude that zeta-tubulin performs a specialized function in basal bodies of differentiated cells, most likely in the function of the basal foot appendage that orients basal bodies with respect to the cell and tissue axes.

P136**Board Number: B242****Intragenic complementation reveals that γ -tubulin has at least three functions important for growth of *Aspergillus nidulans*.**M.A. Hervás-Aguilar¹, K.J. Bichsel¹, B.R. Oakley¹;¹Molecular Biosciences, University of Kansas, Lawrence, KS

γ -tubulin plays a key role in microtubule nucleation at microtubule-organizing centers of both fungal and animal cells, but data from a number of organisms indicate that it has additional important, although less clearly defined, functions. Previously, we created temperature-sensitive mutations through alanine-scanning mutagenesis of the *mipA* (γ -tubulin) gene of *Aspergillus nidulans* and one of these mutations reveals a role for γ -tubulin in inactivating the anaphase promoting complex coupled with Cdh1 at G₁/S. These mutants display a range of phenotypes and we were interested in how many distinct functions were defective in our alleles. One approach to answering this question is through intragenic complementation. The principle underlying intragenic complementation is that if two alleles of a gene that are defective for different functions are co-expressed in the same cell, the protein products of the two alleles will, between them, carry out all the required functions of the protein. Cells in which both alleles are expressed will, thus, grow better than cells in which either of the two alleles is expressed. If the two alleles are defective for the same function, all protein molecules will be defective for that function and there will be little or no improvement in growth. We tested eight conditionally growth inhibited alleles for intragenic complementation by creating strains in which one mutant allele was present at the endogenous *mipA* locus and the same or another mutant allele was inserted at the *wA* locus. All alleles were recessive to the wild-type *mipA* allele. For four alleles, expression of a second copy of the same allele, surprisingly, resulted in improved growth at restrictive temperatures. Several allele pairs exhibited intragenic complementation (defined as a growth advantage compared to two copies of either of the two alleles). The alleles fell into three intragenic complementation groups indicating that γ -tubulin has at least three functions important for growth. To determine if the functions of γ -tubulin could correlate with functional domains in the γ -tubulin, we modeled the structure of *A. nidulans* γ -tubulin and γ -tubulin complex proteins based on the structure of human TUBG1 and GCP4. The results indicate that the two-copy-suppressed alleles cluster together as do each of the intragenic complementation groups. These data indicate that γ -tubulin has at least three functions important for growth that may correlate with functional domains of the protein. Supported by the NIH and the Irving S. Johnson Fund of the KU Endowment. Kyle J. Bichsel is supported by an IRACDA fellowship.*

P137**Board Number: B243****Interaction of Colchicine-Site Drugs with β VI-Isotype of Tubulin.**M. Loew¹, D.L. Sackett², S.L. Bane¹;¹Chemistry, SUNY-Binghamton, Binghamton, NY, ²Program in Physical Biology, NIH/NICHD, Bethesda, MD

Tubulin, the main component of microtubules, exists as multiple isoforms. Although these isoforms are highly conserved, the β VI isoform is significantly divergent in amino acid sequence. This isoform is found in hematologic cells and is recognized as playing a role in platelet biogenesis. Tubulin from the erythrocytes of *Gallus gallus* (Chicken erythrocyte Tubulin, CeTb) contains roughly 95% β VI tubulin. This form of tubulin has been found to lack interaction with colchicine, a feature previously thought to be ubiquitous of tubulin from higher-order eukaryotes. In this study, we sought to gain a better understanding of the structure-activity relationship of the colchicine site of this divergent isoform. We developed a fluorescence-based assay to detect binding of drugs to CeTb and used it to screen known colchicine-site drugs with CeTb. Drugs that showed good affinity for CeTb include podophyllotoxin, combretastatin A2 and A4, and MDL-27048. Colchicine derivatives had poor affinity with the exception of one allocolchicine derivative. Because the colchicine site of human β VI is very similar to that of chicken β VI, these results may have relevance to the action of microtubule-targeting drugs on hematopoietic tissues in humans.

P138**Board Number: B244****Using *Drosophila melanogaster* as a Model Organism to Study Reproductive Toxicants.**K. Hernandez¹, I. Cepin¹, C. Juarez¹, S. Lovell¹, A. Nicholas¹, R. Wint¹, K. . Boekelheide², J. Rollins³;¹Division of Natural Sciences, College of Mount Saint Vincent, Bronx, NY, ²Brown University, Providence, RI, ³College of Mount Saint Vincent, Bronx, NY

There is a growing international movement to make use of the ongoing revolution in biology to revise longstanding approaches to toxicity testing and risk assessment of environmental chemicals, pharmaceuticals, and consumer products. At its core, this new approach will measure molecular changes in model organisms and cells in vitro to develop a mechanistic understanding of chemically-induced alterations in the function of biological pathways. Model organisms, such as *Drosophila melanogaster*, provide the unique advantage of "simplified complexity," portraying complex organismal processes, such as spermatogenesis, without the many redundant proteins and pathways characteristic of mammals. Six different toxicants (pharmaceutical and environmental) were studied: 2,5-hexanedione, carbendazim, taxol, di (2-ethylhexyl) phthalate, colchicine and dibromochloropropane. Three different dosing methods were tested: 1. Adult flies laid eggs in toxicant laced food in order to mimic exposure in young animal, 2. Adult flies were starved and given the toxicant directly by dissolving the toxicant in

sugar water, 3. Embryos were permeabilized and treated with the toxicant directly. Method 1 was the only method that was not lethal and the effect of the toxicant on sperm development was assessed by phase contrast squashes of wild type and β tubulin-GFP flies. Many defects, such as transition to meiosis, meiotic cytokinesis, and sperm elongation and maturation can easily be quantified using this simple assay. DEHP, di (2-ethylhexyl) phthalate showed little effect on sperm development in all three-dose groups. 2,5-hexanedione was lethal at high doses and effected. Flies overexpressing β tubulin by carrying a trans gene of β tubulin-GFP were more sensitive to this toxicant. The wild type exposed to the toxicant had some motile sperm but also showed signs of degenerative spermatocyte cysts. Taxol exposure yielded less motile sperm and shorter sperm tails. Wild type flies exposed to CBZ were more sensitive to the toxicant than β tubulin-GFP flies however little or no effect on sperm development. Future work on this project will be to maximize the efficiency of dosing so lethality will not be an issue and sperm development can be properly assessed using microscopy, fertility and sperm viability assays.

P139

Board Number: B245

Intravital microscopy suggests that induction of mitotic arrest by antimitotic cancer drugs does not predict their efficacy in fibrosarcoma mouse xenograft tumors.

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Despite immense progress in targeted cancer drug design, to date, the most important drugs in chemotherapy remain traditional cytotoxic drugs which are thought to kill proliferating cells, no matter if malignant or not. This "antiproliferative" hypothesis is based on results from the 1970s coming from mouse leukemia models or cell culture experiments.

Microtubule binding drugs are clinically important antiproliferative drugs which arrest cultured cancer cells in mitosis through activation of the spindle assembly checkpoint (SAC). As opposed to empirically found microtubule binding drugs, newer drugs designed to more specifically induce mitotic arrest have repeatedly failed in clinical trials, suggesting that the antitumor effect of microtubule binding drugs is still not well understood. Alas, tissue culture assays are not predictive of their respective clinical success, suggesting that more realistic models are needed to understand why there are such huge differences in antitumor efficacy.

Therefore, we developed an integrated in vivo microscopy and image analysis pipeline of mouse xenografts to compare the cell cycle effects of microtubule drugs to those of newer, targeted antimitotic drugs in the form Ispinesib, an inhibitor of the mitotic kinesin Eg5. Image analysis with a custom developed software for automated three dimensional segmentation and cell cycle classification revealed

that the microtubule interacting drugs Paclitaxel and Eribulin which are in wide clinical use induce similar mitotic arrest as the Eg5 inhibitor Ispinesib which failed clinical trials. Nevertheless, similar to the clinical situation, they seem to be more effective in reducing tumor cell density than Ispinesib in our system. This suggests that, rather than mitotic arrest, a different mechanism, intrinsically coupled to microtubule binding, may be responsible for the antitumor effect of microtubule drugs in vivo.

P140

Board Number: B246

Characterization of a novel microtubule-associated protein in *Toxoplasma gondii*, a human parasite.

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Toxoplasma gondii is a widespread protozoan parasite that can infect nearly all warm-blooded animals. It can cause toxoplasmosis in humans. Infections in immunocompromised individuals and unprotected fetus have devastating consequences, including the development of lethal *Toxoplasma* encephalitis. *T. gondii* is also a model for its relatives in the phylum Apicomplexa, which includes the malarial parasites. These parasites have a highly organized cytoskeleton that is essential to their replication and infection in the host. Furthermore, the cytoskeleton of the parasite has many properties distinct from that in the host cell and therefore an ideal target for anti-parasitic measures. One prominent component of the *T. gondii* cytoskeleton is its set of 22 cortical microtubules. Compared with microtubules found in mammalian cells, the cortical microtubules are highly ordered and exceptionally stable. It is believed that these special properties of the cortical microtubules are due to novel microtubule associated proteins, because the major tubulin subunits in *T. gondii* are essentially identical with those in mammalian cells. In our effort to search for the proteins that regulate the function of the cortical microtubules, we identified a group of proteins that coat the microtubules, including TLAP3 (for TrxL associated protein 3). TLAP3 has no close homolog in the sequenced genomes of mammals, thus is a potential drug target. We have constructed a transgenic line through homologous recombination. In this line, fluorescently tagged TLAP3 is expressed from its endogenous locus. Interestingly, we found that the localization of TLAP3 is restricted to an apical region close to the presumed organizing center of the cortical microtubules. This may suggest an organization role of TLAP3 in the initial construction of the cortical microtubules. Consistent with this hypothesis, TLAP3 is found close to the apex of developing daughter cells. To further test this possibility, we are creating TLAP3 knockout parasite to probe its function using Cre-LoxP mediated excision.

P141**Board Number: B247****What is tubulin glutamylation good for?.**D. Chawla¹, R. Shah¹, J. Lee¹, Z. Barth¹, N. Peel¹;¹Department of Biology, The College of New Jersey, Ewing, NJ

Microtubules are dynamic polymers that play essential structural roles in cell division, vesicle trafficking, and cilia function. Much is known about the regulation of microtubule dynamics by interacting proteins, but the post-translational modification of tubulin as a regulatory mechanism remains relatively unexplored. Tubulin glutamylation is the covalent attachment of glutamic acid to tubulin in the polymerized microtubule. This modification is enriched on long-lived microtubules, and it is proposed that tubulin glutamylation contributes to centriole stability, cilia motility and axon function.

Comprehensive in vivo analyses of the function of tubulin glutamylation have proved challenging because of the existence of a large family of glutamylating enzymes. *C. elegans*, however, has only 5 glutamylating (tubulin tyrosine ligase like; TLL) enzymes making their combinatorial analysis feasible. We have obtained deletion mutations in all five TLL enzymes, non of which display overt phenotypes. To test for redundancy we have combined the mutations and are currently analysing two mutant combinations. First, a triple mutant that shows reduced male fecundity. Second, a quint mutant lacking all glutamylating enzymes. By closely examining the phenotypes of these worms we hope to definitively determine the in vivo function of tubulin glutamylation.

P142**Board Number: B248****Did acetylation of α -tubulin play a role in the evolution of microtubules from filaments?.**R.F. Luduena¹;¹Biochemistry, Univ Texas Hlth Sci Ctr-San Antonio, San Antonio, TX

There is considerable evidence that the first tubulin molecules formed filaments rather than cylindrical microtubules (MTs) (McIntosh et al, Cell 135, 322, 2008; Ludueña, Int. Rev. Cell Mol. Biol. 302, 41, 2013). How could this filament become a microtubule (MT)? There are three factors to consider: 1) Addition of the C-terminus could have favored MT formation; removal of the C-terminus prevents incorporation into MTs (Joe et al, J. Biol. Chem. 284, 4283, 2009). This may be too fortuitous, however. 2) Lateral joining of the protofilaments (PFs) would allow them to exert more force on heavier cargo (McIntosh et al, J. Cell Sci. 123, 3425, 2010). Although this is very likely to have been a factor, it does not explain why a cylinder rather than, say, a triple helix, was favored. 3) Perhaps a more compelling rationale, however, arises from acetylation. This is a very widespread and therefore probably ancient modification of lys40 in α -tubulin that is associated with increased MT stability and intra-luminal structures as well as binding to mitochondria and Golgi (Friedman et al, J. Cell Biol. 190, 363, 2010; Deakin & Turner, J. Cell Biol. 206, 395, 2014). The modified lys40 is in the lumen of the MT. Recent findings suggest that acetylation does

not affect overall MT structure but that it mediates binding to some intra-luminal protein (Howes et al, Mol. Biol. Cell 25, 257, 2014). It is very difficult to imagine the modifying enzyme, the modification, and the associated intra-luminal protein evolving after the MT appeared. These are more likely to have evolved when tubulin formed only PFs and the lumen did not exist. The acetylation could have mediated binding to an ancestor of a present-day intra-luminal protein, or perhaps a protein linking to mitochondria or Golgi. If so, and if that protein was itself a filamentous polymer (Linck et al, J. Biol. Chem. 289, 17427, 2014) then if adjacent tubulin PFs associated with each other at an angle, so as to form a curved rather than a flat ribbon, then it would have been a stronger structure if at least two of the tubulin PFs bound to the ancestral intra-luminal protein. For this the PFs would have had to associate laterally at an angle. At this point, it would have been easy for more PFs to have added laterally until a cylinder appeared. It is unlikely that a cylinder that evolved this way would have been symmetrical, so it is not surprising that there is a seam at the join. It is not necessary for the intra-luminal protein to fill the entire lumen and indeed, once the MT evolved, there was no universal need for an intra-luminal protein at all or even for an acetylated lys40, so these features could have been retained only for specific functions involving intra-luminal proteins.

P143

Board Number: B249

Reconstitution of the human augmin complex provides insights into its architecture and function.

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Proper microtubule nucleation during cell division requires augmin, a microtubule-associated hetero-octameric protein complex. In current models, augmin recruits γ -tubulin, via its hDgt6 subunit's C-terminus, to nucleate microtubules within spindles. However, augmin's biochemical complexity has restricted analysis of its structural organization and function. Here, we reconstitute human augmin and show it is a Y-shaped complex that can adopt multiple conformations. Further, we find that a dimeric sub-complex retains in vitro microtubule binding properties of octameric complexes, but not proper metaphase spindle localization. Addition of octameric augmin complexes to *Xenopus* egg extracts promotes microtubule aster formation, an activity enhanced by Ran-GTP. This activity requires microtubule binding, but not the characterized hDgt6 γ -tubulin-recruitment domain. Tetrameric sub-complexes also induce asters, but activity and microtubule bundling within asters are reduced compared to octameric complexes. Together, our findings shed light on augmin's structural organization, microtubule binding properties and define subunits required for its function in organizing microtubule-based structures.

P144**Board Number: B250****Characterization of human gamma-tubulin isotypes and their differential expression during neuritogenesis.**E. Draberova¹, S. Vinopal¹, V. Sulimenko¹, T. Sulimenko¹, V. Sladkova¹, L. D'Agostino², C.D. Katsetos², L. Kren³, P. Draber¹;¹Biology of Cytoskeleton, Institute of Molecular Genetics AS CR, Prague 4, Czech Republic, ²Pathology Laboratory Medicine, Drexel University College of Medicine, Philadelphia, PA, ³Faculty Hospital Brno, Brno, Czech Republic

Gamma-tubulin is a highly conserved member of the tubulin superfamily essential for microtubule nucleation. Many organisms including humans possess two genes encoding gamma-tubulin, and their coding sequences share a high sequence similarity. It is thought that in mammalian tissues gamma-tubulin 1 represents ubiquitous form of gamma-tubulin, while gamma-tubulin 2 is found predominantly in brain where it is neuron associated. It was suggested that gamma-tubulin 2 could also have some other functions. The molecular basis of the purported functional differences between gamma-tubulins is unknown. Here we have demonstrated that human gamma-tubulins can be discriminated according to their electrophoretic properties and have unraveled through in vitro mutagenesis that the differences originate in the C-terminal regions of the gamma-tubulin molecules. Using epitope mapping we identified an antigenic determinant recognized by an anti-peptide monoclonal antibody that is specific for human gamma-tubulin 1. We have shown both at the mRNA and protein levels, using RT-qPCR and 2D-PAGE, that gamma-tubulin 1 is the predominant isotype in primary neurons derived from human fetal cerebral cortex (ScienCell Research Laboratories). Comparable amounts of both gamma-tubulin isotypes were found in autopsy samples from non-pathologic cerebral cortex of human adults. Localization of gamma-tubulin 1 in mature cortical neurons was confirmed by immunohistochemistry performed on surgically resected clinical samples containing non-lesional cerebral neocortex. Depletion of gamma-tubulin 1 by shRNAi in the human neuroblastoma SH-SY5Y cells resulted in impaired microtubule nucleation and metaphase arrest. A wild-type phenotype in gamma-tubulin 1-depleted cells was restored by expression of exogenous human gamma-tubulin 2. During all-trans retinoic acid-induced neuritogenesis in SH-SY5Y cells, expression of human TUBG2 was upregulated, while the expression of TUBG1 was basically unchanged. Our data suggest that while both human gamma-tubulins are nucleation competent, differences in their properties and accumulation of gamma-tubulin 2 in mature neurons, in the face of a predominant gamma-tubulin 1 expression in these cells, may reflect additional gamma-tubulin 2 function(s) in neurons. This work was supported in part by grants LH12050, NT14467, and M200521203.

Assembly and Disassembly of Cilia/Flagella 1

P145

Board Number: B252

Identification of the molecular ruler that defines the 96-nm repeats of cilia/flagella.

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Primary ciliary dyskinesia (PCD) is an inherited disease caused by abnormal ciliary motility. Majority of PCD cases associated with axonemal disorganization result from mutations in CCDC39 and CCDC40 genes. However, their localizations and functions in cilia/flagella are not clearly resolved. In this study, we revealed the molecular mechanism how FAP59 and FAP172, the *Chlamydomonas* homologues of CCDC39 and CCDC40, govern the assembly of axonemal ultrastructures. Using biochemical pull-down assay and cryo-electron tomography, we showed that FAP59 and FAP172 form a complex, and the absence of the two proteins disrupts 96-nm repeats of axonemes. Structural labeling revealed that FAP59 and FAP172 take 96-nm long extended conformations along outer doublet microtubules, suggesting their possible role as a molecular ruler. To test this hypothesis, we elongated the amino acid sequences of FAP59 and FAP172 by duplicating their coiled-coil domains. Surprisingly, the repeat length became ~128-nm long, and axonemal components such as radial spokes, inner dynein arms, and dynein regulatory complexes were duplicated. These results strongly suggest that the FAP59/172 complex is the molecular ruler that determines the 96-nm repeats in cilia/flagella by regularly recruiting axonemal components to the correct binding sites.

P146

Board Number: B253

TTC26/DYF13/IFT56 transports a potential length sensor as a cargo for controlling the ciliary length.

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Cilia/flagella are microtubule-based organelles that protrude from the surface of most cells, are important to sense extracellular signals and make a driving force for fluid flow. Because the length of cilia/flagella is correlated with their function, cells must have cilia/flagella of adequate length in order to perform their function properly. However, the regulation mechanism of ciliary length is mostly unknown. To control the length of cilia, cells seem to sense ciliary length and to have a feedback pathway to adjust the amount of cargo transport into cilia. The assembly and maintenance of cilia/flagella are dependent on intraflagellar transport (IFT). IFT is an active transport process within cilia

mediated by a bidirectional movement of multiprotein complexes, known as IFT particles. We identified an IFT mutant *Chlamydomonas* strain, *dyf13*, which exhibits short flagella and motility defects. To identify the causes of short flagella, we measured the accumulation of an IFT protein at basal bodies in wild-type and *dyf13* mutant cells. Wild-type cells showed accumulation of IFT at basal bodies that depends on the length of cilia during regeneration. However, the IFT accumulation in *dyf13* mutant cells was always higher than that of wild-type cells and was independent of ciliary length. This observation suggests that DYF13 itself, or its cargo, is related to the feedback pathway and length sensor for ciliary length control.

P147

Board Number: B254

Cilia autonomous regulation of tubulin transport by IFT.

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Microtubules are the major structural elements of cilia and eukaryotic flagella. During ciliary assembly, large amounts of tubulin must be transported into the organelle. Intraflagellar transport (IFT) is the major protein transport system in cilia and IFT has been implicated in tubulin transport. The IFT proteins IFT81 and IFT72, for example, form a module that binds tubulin dimers *in vitro* indicating that IFT particles interact directly with tubulin [1]. Direct *in vivo* imaging of tubulin during transport, however, has proved challenging [2]. To analyze ciliary tubulin transport and its regulation, we expressed GFP- α -tubulin in wild-type *Chlamydomonas reinhardtii*. GFP- α -tubulin was incorporated into cytoplasmic and ciliary microtubules [3]; it was posttranslationally modified and formed heterodimers with β -tubulin suggesting that GFP-tagged and endogenous α -tubulin behave largely similar. Total internal reflection fluorescence (TIRF) microscopy revealed that GFP- α -tubulin entered flagella by active transport and by diffusion. Two-color *in vivo* imaging showed that GFP- α -tubulin moved on IFT trains inside cilia; thus, IFT functions as a tubulin transporter. Without of IFT, GFP- α -tubulin continued to enter cilia by diffusion. To assess how tubulin interacts with IFT, we expressed modified α - and β - GFP-tubulins; this ongoing project revealed C-terminally truncated GFP- α -tubulin is still transported by IFT and assembled into the axoneme. During ciliary growth, IFT-based transport of GFP- α -tubulin was increased $\sim 15x$ over steady-state frequencies and, in growing cilia, the occupancy rate (% of IFT particles carrying GFP- α -tubulin as a cargo) increased from $\sim 10\%$ to $\sim 80\%$ indicating that tubulin transport by IFT is regulated. Fluorescence recovery after photobleaching (FRAP) analysis and western blotting revealed a strong increase in the concentration of soluble GFP- α -tubulin in regenerating over steady-state cilia. In cells possessing both growing and non-growing cilia, tubulin transport via IFT was elevated only in the growing cilia; likewise, the concentration of tubulin was increased solely in the matrix of the growing cilia. Thus, cells can direct tubulin flux specifically into growing cilia and established distinct concentrations of soluble tubulin within different cilia of a given cell. Our data suggest that tubulin transport via IFT is regulated locally in a flagella autonomous manner, likely within the basal body-cilium entity. We propose a model in which IFT functions as a tubulin pump to increase the concentration of soluble tubulin inside cilia, which will

promote the elongation of axonemal microtubules and thereby ciliary growth. 1Bhogaraju et al. 2013. Science 41:1009. 2Hao et al. 2011. Nat Cell Biol 13:790. 3Rasala et al. 2013. Plant J 74:545.

P148

Board Number: B255

A novel *Chlamydomonas* IFT81 mutant reveals that the IFT81 tubulin-binding domain is not crucial for tubulin transport and flagellar assembly.

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Intraflagellar transport (IFT) is a bidirectional motility of IFT complexes A and B along cilia/flagella, driven by the anterograde motor kinesin-II and the retrograde motor cytoplasmic dynein 1b. IFT is important for the assembly and maintenance of cilia/flagella. Here, we report a novel *Chlamydomonas* insertional mutant *ift81-1* in which the hygromycin-resistance gene used for introducing and identifying the mutation has inserted into exon 7 of *IFT81*, resulting in an apparently null mutant. Cells of *ift81-1* fail to assemble flagella; this phenotype is similar to that of *ift74-2*, a null mutation for IFT74, which interacts with IFT81 in IFT complex B (Brown et al., in preparation). Transformation of *ift81-1* with the wild-type IFT81 gene rescued flagellar assembly and function, confirming that the phenotype is due to loss of IFT81. Recently, it was proposed, based primarily on in vitro experiments, that the calponin-homology domain of IFT81 together with the highly basic N-terminal domain of IFT74 forms a tubulin-binding module that is the basis for tubulin transport within cilia (Bhogaraju et al., 2013). The *ift81-1* mutant has provided an opportunity to test this hypothesis in vivo. Within its calponin-homology domain, IFT81 has five highly conserved residues (K73, R75, R85, K112, and R113 in *Chlamydomonas*) which have been implicated in tubulin binding (Bhogaraju et al., 2013). To assess their importance in tubulin transport and flagellar assembly, we rescued *ift81-1* with constructs expressing versions of IFT81 with one or more of these basic residues changed to glutamate. Surprisingly, all the resulting strains, including one in which four out of five of the basic residues were substituted to glutamate (K73R75K112R113/EEEE), had normal IFT and nearly normal length flagella. Substitution of one or two residues (K73R75/EE, R85E, and K112R113/EE) had no effect on flagellar regeneration kinetics, indicating normal tubulin incorporation into the cell's axoneme. More severe mutations (K73R75R85/EEE and K73R75K112R113/EEEE) resulted in slightly slower flagellar regeneration, suggesting a subtle reduction in tubulin incorporation. Because the effects of modifying the putative tubulin-binding residues of IFT81 were mild, we propose that tubulin binding at the IFT81/IFT74 site is mediated primarily by IFT74; alternatively, tubulin transport may be supplemented by diffusion or binding to other IFT-particle proteins.

P149**Board Number: B256****Chlamydomonas IFT43 is essential for the integrity of IFT complex A and required for flagellar assembly.**B. Zhu¹, J. Pan²;¹Tsinghua, Beijing, China, ²School of Life Sciences, Tsinghua University, Beijing, China

The assembly and maintenance of flagella and cilia depends on Intraflagellar transport (IFT). Mutations in the gene encoding IFT43, a subunit of the IFT complex A, cause Sensenbrenner syndrome in humans. In our genetic screens, we find an aflagellate insertional mutant that is disrupted in IFT43, identified through RESDA based PCR cloning strategy. Most mutant cells form clumps of 4 cells within one mother cell wall. After lysis treatment to release the cells from mother cell wall, no cells with flagella could be detected under light microscope. Transformation of the mutants with HA-tagged IFT43 rescued the mutant phenotype, confirming that IFT43 is the causal gene. Thus, IFT43 is essential for flagellar assembly. Similar to other IFT proteins, IFT43 is enriched at the basal body region and present in the flagella. YFP-tagged IFT43 exhibits intraflagellar transport and IFT43 is a part of IFT A complex as evidenced by sucrose gradient analyses and co-immunoprecipitation. IFT43 affects the stability and integrity of IFT-A but not complex B in that the amount of IFT139 in the mutants was reduced and a smaller IFT-A complex was formed in the *ift43* mutant. EM analysis showed that *ift43* mutant has residual flagella that barely protrude out of the flagellar collar. Surprisingly, no IFT particles were found to accumulate in the short flagella revealed by EM in the *ift43* mutants. The possible function of IFT43 in IFT will be discussed.

P150**Board Number: B257****Chlamydomonas MKS1 is required for fully assembly of flagella beyond the transition zone.**W. Chen¹, H. Ho², C. Tsao¹;¹Department of Biological Science and Technology, National University of Tainan, Tainan, Taiwan,²Department of Anatomy, Tzu-Chi University, Hualien, Taiwan

A *Chlamydomonas* mutant strain Lu3 was obtained by insertional mutagenesis and a visual screen for motility defects. Genotyping revealed that the insertion of an exogenous marker in the Lu3 genome causes a deletion of a 60-kb region, which contains 14 genes including flagellar inner dynein arm I1 intermediate chain *IC140* and *Chlamydomonas MKS1* ortholog. *MKS1* is a component of the transition zone complex, and the defect of human *MKS1* leads to Meckel-Gruber Syndrome. The growth of *Chlamydomonas* Lu3 strain showed no difference comparing to the *cw15* reference strain although Lu3 lost normal motility. Unlike *Chlamydomonas cep290* mutant, which lacks a transition zone protein required for integrity of the microtubule-membrane connector at the flagellar base but could still

assemble axonemes (Craigie et al. 2010), morphology of the transition zone in Lu3 cells appeared normal. Lu3 cells failed to assemble full length flagella, and the axonemal doublet microtubule terminated abruptly distal to the transition zone. These observations indicate that *Chlamydomonas MKS1* is required for the fully assembly of axonemes beyond the transition zone but is not essential for the microtubule-membrane connector.

P151

Board Number: B258

Chlamydomonas CC2D2A localizes to the proximal flagellar transition zone and is required for normal flagellar protein content and flagellar assembly.

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The transition zone (TZ), the region at the base of cilia and flagella where the triplet microtubules of the basal body transition into doublets, contains a large number of highly conserved proteins whose cognate genes are often mutated in human ciliopathies. Mutations in *CC2D2A* result in severe ciliopathies characterized by pleiotropic developmental defects and neonatal lethality (Meckel syndrome), or cystic kidney disease, blindness, brain malformation, and mental deficit (Joubert syndrome). In vertebrates, *CC2D2A* has been reported to localize to the TZ and interact with other TZ proteins; however, the specific molecular function of *CC2D2A* is unknown. We are using multiple *Chlamydomonas* TZ mutants to elucidate the functional, structural, and biochemical consequences of disruption of TZ integrity, and we have isolated a mutant harboring an insertion in an exon near the middle of *Chlamydomonas CC2D2A*. The mutant cells have defects in flagellar assembly and fail to hatch out of the mother cell wall following mitosis, but cells grow flagella following digestion of the mother cell wall by autolysin treatment. In each of two rounds of backcrossing to wild-type cells, the mutation segregated 100% with the phenotype, and the phenotype was completely rescued by transformation with a genomic construct encoding HA-tagged *CC2D2A*, indicating that the phenotype results from the mutation in *CC2D2A*. *CC2D2A*-HA localizes to the TZ, slightly proximal to and partially overlapping with the TZ protein CEP290. CEP290, which is required for normal assembly of the microtubule-membrane Y connectors within the TZ, localizes normally within the TZ of the *cc2d2a* mutant; consistent with this, the Y connectors are unperturbed in the *cc2d2a* mutant. Conversely, in the absence of CEP290, *CC2D2A*-HA no longer localizes to the TZ, and instead accumulates on or near the basal bodies, indicating that CEP290 and/or the Y connectors are required to localize *CC2D2A* to the TZ. A third TZ protein, NPHP4, which is located distal to CEP290 in the TZ, is not required to localize either CEP290 or *CC2D2A* to the TZ, and is also dispensable for Y connector formation. A triple-mutant strain, harboring the *cep290*, *nphp4*, and *cc2d2a* mutations, displays slower growth kinetics, indicating that loss of all three proteins might perturb cell cycle progression. Finally, biochemical analysis of isolated *cc2d2a* mutant flagella revealed protein content abnormalities in the membrane + matrix fraction of the isolated flagella, indicating that *CC2D2A*, like CEP290 and NPHP4, is required for establishing and/or maintaining normal flagellar protein content.

P152**Board Number: B259****Architectural variation at the base of the cilia is required for cilia structure and function diversity.**S.C. Jana¹, P. Machado^{1,2}, J. Rocha¹, S. Mendonça¹, S. Werner¹, M. Bettencourt-Dias¹;¹Instituto Gulbenkian de Ciência, Oeiras, Portugal, ²EMBL, Heidelberg, Germany

Cilia are microtubule-based membrane protrusions conserved across evolution involved in cell motility, fluid flow and sensing. The diversity in functions is generally attributed to a core conserved microtubule-based structure, the axoneme, decorated by different structures, membrane and signaling systems. Here we study four classes of cilia that represent very diverse motility and sensory functions within a single organism, the fruit fly. We uncover that the base of the cilia, the basal body and transition zone, is much more diverse than previously thought, showing large variation in number, length, ultrastructure, and connection to other cellular structures. We further demonstrate that basal body diversity is imparted by differential regulation of evolutionarily conserved core components. The tissue specific regulation of core basal body and transition zone genes suggests mechanisms that generate tissue specific phenotypes in human ciliopathic syndromes.

P153**Board Number: B260****Kinesin-2 and kinesin-9 are required for ciliogenesis during rapid development.**E.J. Tomei¹, S.M. Wolniak¹;¹Cell Biology and Molecular Genetics, University of Maryland, College Park, MD

Spermatogenesis in the semi-aquatic fern, *Marsilea vestita*, is a rapid, synchronous process that is initiated when dry microspores are placed in water. Development is post-transcriptionally driven and can be divided into two phases. The first phase consists of a series of mitotic divisions that produce 7 sterile cells and 32 spermatids. During the second phase, each spermatid differentiates into a corkscrew-shaped motile spermatozoid with ~144 cilia. Ciliogenesis occurs on basal bodies that are positioned in two rows at regular intervals along the dorsal face of a coiled microtubule ribbon. Kinesin motor proteins are known to participate in both mitosis and ciliogenesis and provide the unique opportunity to study the mechanisms that regulate both stages of development using one class of proteins. Through RNAseq, we assembled a transcriptome that shows the abundance of transcripts throughout development. Over 150 kinesin-like sequences were identified in this transcriptome that represent at least 57 unique kinesin transcripts. Based on alignments of the kinesin motor domain, members of the kinesin-2, -4/10, -5, -7, -8, -9, -13, -14, -15, -16, -18, and -19 families as well as several orphan kinesins were found. This complement of kinesins is similar to other plants that make ciliated spermatozoids, such as *P. patens*. In *M. vestita* most of the kinesin transcripts (84%) change in abundance during gametophyte development. Kinesin mRNAs that decrease in abundance (44%) encode proteins thought to be involved in mitosis, while those that increase in abundance during development (40%) have

proposed roles in ciliogenesis. Kinesin-2 and -9 are only found in ciliated organisms, and the abundance of these transcripts increases ~12-fold during later stages of spermatogenesis. This increase in transcript abundance is independent of transcription and is instead results from the unmasking and processing of stored pre-mRNAs. RNAi knockdowns show that kinesin-2 is required for the later divisions of spermatogenous cells and that both kinesin-2 and -9 are required for *de novo* basal body formation and ciliogenesis. Without kinesin-2, most spermatozooids contain two or more coiled microtubule ribbons with attached cilia and very large cell bodies. Those that emerge from the spore have cilia that are substantially longer than controls. Kinesin-9 knockdowns have basal bodies that are irregularly positioned and cilia are not localized in the anterior portion of the spermatozoid. Spermatozoid swimming behavior in kinesin-2 and -9 knockdowns is altered because of resultant defects in ciliogenesis. Kinesin-2 knockdowns can only swim or quiver in place while kinesin-9 knockdowns swim erratically compared to controls. (Supported by NSF grant MCB-0842525 to SMW).

P154

Board Number: B261

Meckelin guides orientation of basal bodies along the striated rootlet.

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Meckelin (MKS3) is a protein of the primary cilia transition zone that functions in ciliogenesis and ciliary gating. MKS3 appears to have similar functions and location in *Paramecium tetraurelia* since FLAG-MKS3 is found associated with and slightly above each basal body and RNAi for MKS3 leads to loss of cilia. However, RNAi for MKS3 also leads to the disorganization of rows of basal bodies that normally run from anterior to posterior. In the areas of misalignments, the basal bodies with their post ciliary and transverse rootlets are found rotated and out of their rows. However, the rootlets are attached to the basal bodies at the expected angles relative to each other. We propose that MKS3 guides new basal bodies as they move toward the anterior of the cell along the striated rootlet (SR) of the parent basal body. These SRs are aligned along the rows of basal bodies; new basal bodies do not yet have their own SR. The loss of MKS3 results in loss of interactions between the new basal body and the parent basal body SR. Without a guide to maintain orientation, the new basal bodies migrate out their rows and out of line and, when they form their SRs, these rootlets do not project toward the anterior as expected. To test for interactions of MKS3 with SR components, we first identified potential SR proteins, expressed them with epitope tags and determined whether they were located in the SR. Of the 24 potential SR protein genes, 13 were selected as representative of sets of paralogs for epitope tagging. Nine of them were associated with the SR, often with non-uniform distributions. Those sequences with SF assemblin domains (similar to those in the *Chlamydomonas* rootlet proteins) coded for proteins that we found in the *Paramecium* SRs; conversely those without this domain were not in the rootlets. MKS3 interacted sufficiently (directly or indirectly) with the striated rootlet proteins to pull them down with a GST-fusion of the 252 C-terminal residues of the *Paramecium* MKS3. We thank J. Beisson for the anti-SR antibody. P20 GM103449; P30 GM103498; R01 GM 59988

P155**Board Number: B262****Asymmetrically localized Fop1 stabilizes basal bodies.**B.A. Bayless¹, T. Giddings², I.M. Cheeseman³, C.G. Pearson⁴;¹Cells, Stem Cells and Development, University of Colorado- Anschutz Medical Center, Aurora, CO,²Molecular, Cellular and Developmental Biology, University of Colorado at Boulder, Boulder, CO,³Whitehead Inst Biomed Res, Cambridge, MA, ⁴Department of Cell and Developmental Biology, University of Colorado-Anschutz Medical Center, Aurora, CO

Motile cilia are necessary for directed extracellular fluid flow. Basal bodies position the cilium and anchor the mechanical forces produced by ciliary beating. The basal body is made up of a set of nine triplet microtubule blades arranged in a cylinder. These microtubules are somehow stabilized to maintain the basal body during ciliary beating. The ciliary beat pattern is asymmetric, a unidirectional power stroke is followed by a refractory stroke which occurs along the same axis. The bulk of the force that the basal body experiences from ciliary beating likely occurs along this axis. The basal body assembly factor Bld10/Cep135 is also necessary to stabilize basal bodies, in *bld10Δ* cells, triplet microtubules are unstable and their disassembly initiates at the triplet microtubules along the axis of the cilium beat stroke. Inhibiting ciliary beating rescues the basal body disassembly phenotype suggesting that Bld10 stabilizes the triplet microtubules from the forces of ciliary beating. Poc1 and Fop1 interact with Bld10, stabilize basal bodies, and localize along the entire length of the basal body. We examined the relationship between Poc1 and Fop1, and find that Poc1 is necessary for the temporal loading of Fop1 into the basal body. Interestingly, Fop1 localizes asymmetrically to the basal body at triplet microtubules that align with the axis of the ciliary beat stroke. We hypothesize that Fop1 helps to stabilize the triplet microtubules on the sides of the basal body that experience the most force from ciliary beating. Taken together, our data suggest that Bld10, Fop1 and Poc1 are stability factors for the basal body and Poc1 helps to load Fop1 asymmetrically along the triplet microtubules.

P156**Board Number: B263****SPEF1 regulates the length of central microtubules in 9+2 motile cilia.**M. Guha¹;¹Cellular Biology, The University of Georgia, Athens, GA

SPEF1 is a microtubule-binding protein with a calponin homology (CH) domain, and structural similarity to the End Binding (EB) plus end tracking proteins. SPEF1 homologs are present in most ciliated organisms. The ciliate *Tetrahymena thermophila* has two paralogs of SPEF1. Cells lacking both of these paralogs swim very slowly and have fewer but normal length cilia. Ultrastructural studies revealed a mixture of normal 9+2 and abnormal 9+1 or 9+0 axoneme cross-sections, indicating defects in the assembly of central microtubules. Longitudinal sections revealed that frequently the proximal portions of the central microtubules (near the minus ends) were truncated. Purified SPEF1 bound to microtubules

in a sedimentation assay. Phylogenetic studies revealed that SPEF1 is absent in species with cilia naturally lacking central microtubules. Taken together, these results suggest that SPEF1 regulates the length of the central microtubules; possibly by affecting the stability of their minus ends. Interestingly, a knockout strain lacking a conserved central pair protein, PF20, also has fewer cilia. Thus, in *Tetrahymena* defects in the central apparatus are associated with reduced ciliogenesis.

P157

Board Number: B264

Cep164, but not EB1, is essential for centriolar localization of TTBK2 and its function in ciliogenesis.

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Primary cilia are microtubule-based antenna-like organelles that emanates from the mother centriole-derived basal body in most quiescent mammalian cells. Primary cilia transduce chemical and mechanical signals from the extracellular milieu and play critical roles in the development and homeostasis of many tissues. Defects in formation and/or function of primary cilia are associated with many human diseases, including polycystic kidney disease, retinal degeneration and neurological defects. In ciliogenesis, a centriolar protein CP110 is removed from the distal region of the mother centriole, which allows the mother centriole to move and dock to the plasma membrane via the distal appendages of the mother centriole and to nucleate the ciliary axoneme. CP110 and its interactor Cep97 suppress ciliogenesis by blocking axonemal microtubule assembly; therefore, removal of CP110 from the mother centriole is required for initiating axoneme extension and ciliogenesis. Tau tubulin kinase-2 (TTBK2) has an essential role in initiation of ciliogenesis by promoting CP110 removal and recruitment of intraflagellar transport (IFT) protein complexes. When cells are growth-arrested by serum starvation, TTBK2 is recruited to the distal region of the mother centriole. Therefore, the centriolar localization of TTBK2 is a key step in initiation of ciliogenesis. Previous studies demonstrated that TTBK2 binds to EB1 and Cep164, both of which are essential for ciliogenesis. However, the roles of the binding of these proteins in ciliogenesis and centriolar localization of TTBK2 have remained unclear. To elucidate the mechanisms underlying centriolar localization of TTBK2, we analyzed the roles of EB1 and Cep164 in TTBK2 localization. TTBK2 bound to EB1 and Cep164 through two SxIP motifs and a proline-rich motif in the C-terminal region of TTBK2, respectively. TTBK2 mutants at SxIP motifs did not localize at the plus-ends of microtubules, but localized at the mother centriole, and they rescued the defect in ciliogenesis upon TTBK2 knockdown. These results suggest that EB1 is not essential for centriolar localization of TTBK2. In contrast, depletion of Cep164 or overexpression of Cep164-N, which can bind to TTBK2 but not localize on the centriole, suppressed centriolar localization of TTBK2. Furthermore, non-Cep164-binding mutants of TTBK2 at the proline-rich motif did not rescue the defects in ciliogenesis and CP110 removal upon TTBK2 knockdown. These results suggest that Cep164, but not EB1 is essential for centriolar localization of TTBK2 in the initiation of ciliogenesis. We also showed that TTBK2 phosphorylates Cep164 and Cep97 and inhibits the interaction between Cep164 and its binding protein Dishevelled-3.

P158**Board Number: B265****The G-domain of the Joubert syndrome protein ARL13B is required for anchoring to the axoneme and uniform distribution along the cilium.**E. Revenkova¹, G.L. Gusella², T. Tedeschi¹, C. Iomini¹;¹Department of Ophthalmology, Icahn School of Medicine at Mount Sinai, New York, NY, ²Department of Medicine, Icahn School of Medicine at Mount Sinai, New York, NY

Cilia-mediated signal transduction involves precise targeting and localization of selected molecules along the ciliary membrane, but the molecular mechanism underlying these events is unclear. ARL13B is a membrane-associated small G-protein that localizes along the cilium and functions in ciliary protein transport and signaling. In human, mutations of ARL13B cause Joubert syndrome, and in model organisms ARL13B deficiencies lead to structural defects in cilia and mislocalization of ciliary proteins.

To gain mechanistic insight into ARL13B activity and positioning along the ciliary membrane, we sought to identify interaction partners essential for its function in the cilium. Using a tandem affinity purification (TAP) approach and mass spectrometry, we found that the major proteins co-purifying with TAP-tagged ARL13B from ciliated RPE cells were alpha- and beta-tubulins. This interaction was confirmed by immunoprecipitation of both GST-tagged and endogenous ARL13B from cell extracts. Moreover, GST pull-down assay using a mixture of purified GST-ARL13B and purified tubulin demonstrated that the interaction with tubulin is direct. Mapping of the tubulin-interacting regions of ARL13B revealed that the G-domain and significantly truncated fragments of the G-domain, but not the coiled-coil region, interact with soluble tubulin.

To address the function of the G-domain within the ciliary compartment, we obtained an ARL13B mutant lacking the G-domain (ARL13B- Δ GD) but retaining the ciliary localization. In wild-type mouse embryonic fibroblasts (MEFs), wild-type ARL13B or mutant ARL13B- Δ GD, which were expressed as GFP fusions using lentiviral vectors, followed the distribution of the endogenous ARL13B along the whole cilium. In contrast to the even distribution in wild-type cells, in *Arl13b* null (*hnn*) MEFs ARL13B- Δ GD accumulated at the distal tip and dramatically diminished along the cilium. While the overexpression of ARL13B- Δ GD caused cilium elongation in wild-type cells, it failed to rescue shortened cilia in *hnn* MEFs.

To test whether ARL13B G-domain is required for anchoring ARL13B to the ciliary axoneme, we performed in vitro pull down of GST-ARL13B fragments using axonemes purified from *Chlamydomonas* flagella. The assay showed that both full-length ARL13B and the G-domain bind isolated axonemes, while the ARL13B coil-coil domain does not, indicating that ARL13B interacts with axoneme through its G-domain capable of binding to tubulin.

We conclude that the G-domain of ARL13B is not required for trafficking to the cilium but it is indispensable for proper axonemal distribution of ARL13B.

P159**Board Number: B266****Primary cilia in neural progenitors are regulated by neutral sphingomyelinase 2 and ceramide.**

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We show here that human embryonic stem (ES) and induced pluripotent stem (iPS) cell - derived neuroprogenitors (NPs) develop primary cilia. Ciliogenesis depends on the sphingolipid ceramide and its interaction with atypical PKC (aPKC), both of which distribute to the primary cilium and the apicolateral cell membrane in NP rosettes. Neural differentiation of human ES cells to NPs is concurrent with a 3-fold elevation of ceramide, in particular saturated, long chain C16:0 ceramide (N-palmitoyl sphingosine) and non-saturated, very long chain C24:1 ceramide (Nnervonoyl sphingosine). Decreasing ceramide levels by inhibiting ceramide synthase or neutral sphingomyelinase 2 (nSMase2) leads to translocation of membrane-bound aPKC to the cytosol, concurrent with its activation and the phosphorylation of its substrate Aurora kinase A (AurA). Inhibition of aPKC, AurA, or a downstream target of AurA, HDAC6, restores ciliogenesis in ceramide-depleted cells. Importantly, addition of exogenous C24:1 ceramide reestablishes membrane association of aPKC, restores primary cilia, and accelerates neuronal process formation. Taken together, these results suggest that ceramide prevents activation of HDAC6 by cytosolic aPKC and AurA, which promotes acetylation of tubulin in primary cilia and potentially, neuronal processes. This is the first report on the critical role for ceramide generated by nSMase2 in stem cell ciliogenesis and differentiation. (Supported by grant NSF00028 and AHASE00089)

P160**Board Number: B267****A new step in ciliogenesis: The ANO1 Nimbus.**

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Many cells possess a single, non-motile, primary cilium that is thought to be a sensory transducer akin to a cellular antenna. Ciliogenesis involves migration of the basal body to the cell surface followed by outgrowth of the axoneme by intraflagellar transport. We have identified another early step in cilium development, formation of a novel structure - the *nimbus*. Prior to cilia extension, ANO1, a Ca²⁺-activated Cl⁻ channel, is organized into a torus-shaped structure along with other ciliary proteins including the small GTPases CDC42 and Arl13b, exocyst complex components, and acetylated α -tubulin and γ -tubulin. Furthermore, we have identified this region as an area of RNA localization. This structure we call the *nimbus* forms an interface between the microtubule cytoskeleton of the nascent cilium and

the surrounding cortical actin cytoskeleton. During ciliogenesis, the nimbus disassembles and ciliary components, including ANO1, move into the cilium. Our data support a model where the nimbus provides a scaffold for staging of ciliary components and cilia assembly and expands to include the potential role of RNA localization in cilium formation.

P161

Board Number: B268

Actin and microtubule-interacting protein Girdin promotes ciliogenesis by regulating basal body positioning.

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Primary cilia are present on the surface of most cell types in metazoans and have recently emerged as key mediators of diverse signaling pathways critical for embryonic development and adult tissue homeostasis. Cilia from all studied organisms share a conserved core organization comprised of a microtubule-based axoneme surrounded by a specialized membrane. Primary cilium assembly is a multi-step process that relies on mother centriole-to-basal body (BB) maturation, BB migration and anchoring to membrane, establishment of the 'ciliary gate', and axoneme elongation from the BB via intraflagellar transport (IFT) of ciliary components. Although actin and microtubule networks are pivotal for ciliogenesis, the molecular mechanisms regulating cytoskeletal dynamics during different steps of cilia biogenesis are not well understood. We find that the actin and microtubule-interacting protein Girdin localizes to the BB in *C. elegans* sensory neurons and mammalian cells. Loss of *girdin* function in *C. elegans* results in defective sensory cilia morphology, ultrastructure, and localization of BB and transition zone proteins. Similarly, Girdin knockdown in mammalian cells leads to loss of, or shortened cilia. Analysis of cytoskeletal organization shows that both actin and microtubule networks may be disrupted in mammalian cells upon Girdin knockdown, leading to altered BB positioning and decreased IFT accumulation at the ciliary base. Mammalian Girdin is known to couple extracellular cues to actin dynamics during cell migration. Our preliminary findings suggest that it may also coordinate cytoskeletal dynamics to ensure proper BB positioning and cilia assembly. We will present our ongoing efforts to further characterize a novel role for this highly conserved protein in primary cilia biogenesis and function in *C. elegans* sensory neurons and mammalian cells.

Centrosome Assembly and Functions 1

P162

Board Number: B270

The centrosome is also an actin-organizing center, ATOC.

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The centrosome is the main microtubule-organizing centre. It is involved in the regulation of cell polarity, cell motility and cell division. Although centrosome functions mainly depend on microtubules, the actin network has been shown to be involved in several key centrosome activities such as centrosome positioning at the cell centre, membrane docking during ciliogenesis or immune synapse formation as well as mitotic spindle assembly and positioning. However, the centrosome-actin connection has remained elusive. Actin and actin-binding proteins have been detected at the centrosome by proteomic analyses and immuno-stainings, but experimental data demonstrating the capacity of centrosome to organize actin filaments and interact with the actin network are still missing.

Here, we provide the first compelling evidence that centrosomes assemble and anchor actin filaments. Centrosomes were isolated from human T lymphocytes expressing eGFP-centrin. As expected, they induced the nucleation of microtubules in the presence of purified tubulin dimers, but they also promoted the assembly of large radial arrays of actin filaments in the presence of actin monomers. These networks appeared to stem from short filaments, stably and tightly bound to the purified centrosomes. These filaments could not be disassembled by actin depolymerizing drugs. Highly stable actin filaments were also present at the centrosome of living cells. Centrosome displayed the same capacity to promote the growth of long filaments in the presence of purified actin monomers after removal of cell membrane. The actin organizing property of centrosome in living cells was further demonstrated by time-lapse monitoring of lymphocytes, in which a cytoplasmic actin cloud permanently accompanied the moving centrosome.

These results point toward a new role for the centrosome as an actin-organizing centre. They shine some light on numerous prior observations of centrosome and actin network mutually influencing each other.

P163**Board Number: B271****Centrosomes promote efficient spindle assembly, maintain cell viability, and help orient spindles in epithelial tissue during development.**J.S. Poulton¹, J.C. Cuningham¹, M. Peifer¹;¹Department of Biology, University North Carolina, Chapel Hill, NC

Mitotic spindles are critical for accurate chromosome segregation. Centrosomes, the primary microtubule nucleating centers of animal cells, play key roles in forming and orienting mitotic spindles. However, the survival of *Drosophila* without centrosomes suggested they are dispensable in somatic cells, challenging the canonical view. We used fly wing disc epithelia as a model to resolve these conflicting hypotheses, revealing that centrosomes play vital roles in spindle assembly, function, and orientation. Many acentrosomal cells exhibit prolonged spindle assembly, chromosome mis-segregation, DNA damage, misoriented divisions, and eventual apoptosis. We found that multiple mechanisms buffer the effects of centrosome loss, including alternative microtubule nucleation pathways (Augmin and RanGTP) and the Spindle Assembly Checkpoint (SAC). Apoptosis of acentrosomal cells is mediated by JNK signaling, which also drives compensatory proliferation to maintain tissue integrity and viability. These data reveal the importance of centrosomes in fly epithelia, but also demonstrate the robust compensatory mechanisms at the cellular and organismal level. Interestingly, these mechanisms are insufficient when we disrupt the SAC in acentrosomal cells. We are currently exploring how this affects brain development, which is grossly perturbed in the absence of both centrosomes and the SAC, and may serve as a model for microcephaly.

P164**Board Number: B272****The centrosome as a phoenix organelle: burned by its own proteasome during fever and revived by its own molecular chaperone Hsp70.**S. Doxsey¹, A. Vertii²;¹Univ Massachusetts Med Sch, Worcester, MA, ²PMM, Univ Massachusetts Med Sch, Worcester, MA

The centrosome is a non-membranous organelle that performs many essential functions including microtubule nucleation/organization, ciliogenesis, cell division, membrane trafficking, and immunological synapse formation. The immune response during inflammation is often accompanied by elevated body temperature. Although the detrimental effect of heat shock on centrosome is established, the physiological relevance and mechanisms of centrosome damage remain elusive. Here we provide evidence that the febrile condition results in centrosome damage in patients' leukocytes. We find that local proteasome machinery at the centrosome is responsible for centrosome damage and functional inactivation of centrosome substructures. This heat-induced centrosome degradation pathway appears to be unique to the centrosome, as another non-membranous organelle, the midbody,

remains unchanged following heat shock. Forced targeting of the molecular chaperone, Hsp70-GFP, to the centrosome prevents centrosome inactivation, whereas centrosome-excluded Hsp70 or chaperone-inactive mutants fail to protect the centrosome. This novel mechanism of centrosome regulation during fever is an important aspect of the previously demonstrated role of centrosomes in the immune response.

P165

Board Number: B273

Plk1 controls PCM assembly by phosphorylating SPD-2 and SPD-5 in *C. elegans*.

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Centrosomes, the major microtubule organizing centers in animal cells, are composed of a centriole pair surrounded by a matrix of pericentriolar material (PCM) that nucleates and anchors microtubules. Centrioles direct assembly of the PCM, which expands during mitotic entry in a process termed centrosome maturation; the expanded PCM is subsequently disassembled during mitotic exit. The mechanisms that restrict PCM assembly around centrioles and regulate its cell cycle dynamics remain poorly understood. Here we use the *C. elegans* embryo to analyze the regulation of PCM assembly. Genome-wide screens have identified 4 *C. elegans* proteins that are required for PCM assembly. SPD-5 is a large coiled-coil protein that is thought to be the major component of the PCM matrix. PCM assembly also requires SPD-2, Aurora A, and the polo family kinase Plk1. We confirmed the essential role of Plk1 in PCM assembly by constructing *C. elegans* strains in which the only source of PLK-1 was a single copy transgene expressing either wild-type PLK-1 (PLK-1WT) or an analog-sensitive “Shokat” mutant (PLK-1AS), in which the catalytic pocket was mutated to accept bulky purine analogs and enable kinase inhibition by the cell-permeable PP1 analog 1NM-PP1. Strains also included a transgene expressing the centrosomal marker GFP:: γ -tubulin to monitor the PCM. Addition of 1NM-PP1 to permeabilized embryos during S-phase prior to nuclear envelope breakdown abolished mitotic PCM expansion in embryos carrying the plk-1AS, but not the plk-1WT, transgene. Addition of 1NM-PP1 to embryos arrested in metaphase with a proteasome inhibitor also led to rapid disassembly of the mitotic PCM in embryos carrying the plk-1AS, but not the plk-1WT, transgene, indicating that Plk1 activity is essential for both establishment and maintenance of mitotic PCM. To understand how Plk1 contributes to PCM assembly, we are analyzing the consequences of mutating all predicted Plk1 sites that are conserved among nematodes, either individually or in localized groups, in SPD-2 (7 sites) and SPD-5 (25 sites). We have already identified 2 Plk1 sites in SPD-2 and 2 sites in SPD-5 that are essential for PCM assembly, suggesting that Plk1 regulates SPD-5 polymerization by phosphorylating both SPD-2 and SPD-5.

P166**Board Number: B274****Regulated assembly of a supramolecular centrosome scaffold *in vitro*.**J. Woodruff¹, O. Wueseke¹, V. Viscardi², J. Mahamid³, K. Oegema², A. Hyman¹;¹Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany, ²Ludwig Institute for Cancer Research, La Jolla, CA, ³Max Planck Institute of Biochemistry, Martinsried, Germany

A centrosome comprises a pair of centrioles surrounded by an amorphous protein mass called the pericentriolar material (PCM). Despite its importance as major microtubule-organizing center, the mechanism of PCM assembly and its regulation are not understood. In a *C. elegans* embryo, the coiled-coil protein SPD-5 is essential for PCM assembly. We found that recombinant SPD-5 can polymerize to form micrometer-sized porous networks *in vitro*. Network assembly was accelerated by two conserved regulators that control PCM assembly *in vivo*, Polo-like kinase-1 and SPD-2/Cep192. Interestingly, only the assembled SPD-5 networks, and not unassembled SPD-5 protein, functioned as a scaffold for other PCM proteins. Our results suggest that PCM size and binding capacity emerge from the regulated formation of a porous network from one coiled-coil protein. Furthermore, these results suggest that PCM growth is autocatalytic, such that polymerized SPD-5 can recruit additional SPD-5 and its own positive regulators, leading to rapid network expansion.

P167**Board Number: B275****Plk1 binds to the two distinct phospho-motifs on a centrosomal scaffold, Cep192, to promote bipolar spindle formation.**L. Meng¹, J. Park², T. Kim², K. Lee²;¹National Institute of Health, Bethesda, MD, ²NIH/NCI, Bethesda, MD

Serving as microtubule (MT)-organizing centers, centrosomes play a key role in bipolar spindle formation during mitosis. A centrosomal scaffold protein, Cep192, is required for proper centrosome organization and bipolar spindle assembly. However, how it promotes these events remains largely unknown. A recent study with *Xenopus* extracts suggested that Plx1 interacts with the p-T46 motif of Cep192 to form a Cep192-dependent AurA-Plx1 cascade critical for the establishment of bipolar spindles. Here we demonstrated that, in cultured human cells, Cep192 recruits AurA and Plk1 in a cooperative manner and this event is important to promote the reciprocal activation of the latter two enzymes at centrosomes. Strikingly, Plk1 interacted with human Cep192 through two distinct phospho-motifs—the p-T44 motif (which is analogous to the *Xenopus* p-T46 motif) and a yet uncharacterized p-S995 motif—in a manner that requires its C-terminal noncatalytic polo-box domain (PBD). Intriguingly, the interaction of Plk1 with the p-T44 motif required the presence of AurA, whereas the interaction with the p-T995 motif occurred only in the absence of AurA, suggesting that Plk1 binds to Cep192 in two biochemically distinguishable modes. Consistent with this view, loss of either one of these interactions induced a mild defect in bipolar spindle formation and mitotic progression as a result of improper recruitment of Plk1 and γ -

tubulin to centrosomes. Under the same conditions, elimination of both interactions induced a greatly enhanced defect in these processes, suggesting that the defect associated with the loss of T44- and S995-dependent interactions is additive. Taken together, we propose that Plk1 forms two functionally non-overlapping complexes with Cep192 to promote centrosome-based bipolar spindle formation and therefore proper M-phase progression.

P168

Board Number: B276

Measuring the in-vivo Activity of Separase at Centrosome.

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Separase is best known for its function in sister chromatid separation at the metaphase-anaphase transition. It also has a role in centriole disengagement in late mitosis/G1. To gain insight into the activity of separase at centrosomes, we developed two separase activity sensors: mCherry-Scc1^{(142-467)- Δ NLS}-eGFP-PACT and mCherry-kendrin⁽²⁰⁵⁹⁻²³⁹⁸⁾-eGFP-PACT. Both localize to the centrosomes and enabled us to monitor local separase activity at the centrosome in real time. Both centrosomal sensors were cleaved by separase before anaphase onset, earlier than the corresponding H2B-mCherry-Scc1⁽¹⁴²⁻⁴⁶⁷⁾-eGFP sensor at chromosomes. This indicates that substrate cleavage by separase is not synchronous in the cells. Depletion of the proteins astrin or Aki1, which have been described as inhibitors of centrosomal separase, did not lead to a significant activation of separase at centrosomes, emphasizing the importance of direct separase activity measurements at the centrosomes. Inhibition of polo-like kinase Plk1, on the other hand, decreased the separase activity towards the Scc1 but not the kendrin reporter. Together these findings indicate that Plk1 regulates separase activity at the level of substrate affinity at centrosomes and may explain in part the role of Plk1 in centriole disengagement.

P169

Board Number: B277

PLK1 phosphorylation is essential for the separase-dependent cleavage of pericentrin during mitotic exit.

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The centrosome duplication events are tightly linked to the cell cycle. In order to duplicate in the next cell cycle, mother and daughter centrioles should be disengaged at the end of mitosis. Therefore, centriole disengagement is considered a licensing step for centriole duplication. Separase is known to play an essential role in the centriole disengagement. We and others identified pericentrin as a substrate of separase. Here, we report the importance of PLK1 phosphorylation in the separase-dependent cleavage of pericentrin. PLK1 phosphorylates multiple sites of pericentrin during mitotic

entry. We observed that separase could not cleave the phospho-resistant mutants of pericentrin during mitotic exit. Furthermore, centriole disengagement was inhibited in the cells rescued with the phospho-resistant mutants of pericentrin. Based on these results, we propose that PLK1 phosphorylation is a prerequisite step for separase-dependent cleavage of pericentrin and eventually for centriole disengagement.

P170

Board Number: B278

The RNA-binding Protein ATX-2 Interacts with SZY-20, and Opposes ZYG-1 to limit Centrosome Size.

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Centrosomes are critical sites for controlling microtubule dynamics, and exhibit dynamic changes in size during the cell cycle. As cells progress to mitosis, centrosomes recruit more microtubules to form bipolar spindles. The *szy-20* gene encodes a centrosome-associated RNA-binding protein that negatively regulates ZYG-1, a key centrosome duplication factor. It has been shown that *szy-20* mutants possess enlarged centrosomes, causing abnormal microtubule processes and embryonic lethality. SZY-20 contains putative RNA-binding domains; mutating these domains perturbs RNA-binding by SZY-20 in vitro and its capacity to regulate centrosome size in vivo. To further elucidate the roles of SZY-20 and RNA-binding in regulating centrosome assembly and size, we sought to identify factors associated with SZY-20. By proteomics, we identified an RNA-binding protein ATX-2 reproducibly pulled-down with SZY-20. Consistent with its physical association, depleting ATX-2 produces embryonic lethality and cell division defects seen in *szy-20(bs52)* embryos, including a failure of cytokinesis and polar body extrusion, and abnormal spindle positioning. Thus, ATX-2 is required for proper cell divisions in which *atx-2* acts synergistically with *szy-20*: Knocking down ATX-2 in *szy-20(bs52)* mutants enhanced the embryonic lethality and cell division defects. Genetic analyses indicate that not only *szy-20* but also *atx-2* negatively regulates centrosome assembly. *atx-2(RNAi)* partially restores centrosome duplication to *zyg-1(it25)* embryos. Knocking down both *szy-20* and *atx-2* almost completely restored bipolar spindle formation to *zyg-1(it25)* embryos, suggesting a positive interaction between *atx-2* and *szy-20* in regulating centrosome assembly. Intriguingly, ATX-2-depleted embryos exhibit enlarged centrosomes as shown in *szy-20(bs52)*. Embryos partially depleted of ATX-2 possess significantly increased ZYG-1 and pericentriolar materials at centrosomes. Enlarged centrosomes are more evident in a null allele *atx-2(ne4297)* embryos, suggesting an inverse correlation between the amount of ATX-2 and centrosome size. Quantitative western and immunostaining analyses show that ATX-2 is nearly absent in *szy-20(bs52)*, much similarly in *atx-2(ne4297)*, but unaffected in *zyg-1* mutants. In contrast, SZY-20 levels are unaffected in *atx-2* or *zyg-1* mutants. Thus, SZY-20 acts upstream of ATX-2 and promotes the level of ATX-2, which opposes ZYG-1 to limit centrosome size. In this study we identify ATX-2 as a negative regulator of centrosome assembly and size.

P171**Board Number: B279****Determining the function of the protein PCID2 at the centrosome.**N. Schramm¹, K. Resendes¹;¹Biology, Westminster College, New Wilmington, PA

Human PCID2 is a member of the TREX2 complex which associates with the nuclear pore basket to facilitate mRNA export. Multiple components of the TREX2 complex, including PCID2, serve additional roles in the cell other than in nuclear mRNA export. Specifically, PCID2 and centrin 2 co-localize outside of the nucleus at the centrosome. Recent characterization indicates that PCID2 is present at the centrosome during the G1/S and G2/M phases of the cell cycle. The localization of Centrin 2 at the centrosome in those phases is linked to its role as required factor for centriole duplication. While the relationship between PCID2 and centrin 2 at the centrosome during these periods is not yet fully understood, we have previously shown that siRNA knockdown of centrin 2 disrupts centrosomal localization of PCID2. In contrast, PCID2 knockdown does not affect the positioning of centrin 2. Because characterization of PCID2 in human cells thus far has been limited to its roles in nuclear mRNA and protein export investigating its significance outside of the nucleus is crucial to understanding its overall role in the cell and its functional relevance at the centrosome. We are performing both siRNA knockdown of PCID2 as well as overexpression of a GFP-PCID2 recombinant plasmid to determine the effects of altering PCID2 expression levels on the centrosome. Because the PCI domain contained within PCID2 typically serves as a structurally stabilizing scaffolding unit within large complexes, it is expected that increased or decreased levels of PCID2 will produce an observable effect on positioning and assembly of the centrosome due to abnormal protein stoichiometry. We are currently testing this prediction by assessing changes in centrosome number, position and mitotic defects such as altered spindle organization via fluorescence microscopy.

P172**Board Number: B280****The role of mouse CKAP2 phosphorylation in centrosome biogenesis.**B. Yoo¹, C. Park¹, H. Kim¹, J. Park¹, C. Bae¹;¹Sungkyunkwan Univ Sch Med, Suwon, Korea

CKAP2, cytoskeleton-associated protein, is regulated during the cell cycle, with the protein being degraded in late mitosis in an APC/C-Cdh1 dependent manner. Our previous studies have suggested that cellular function of CKAP2 pertain to regulation of the mitotic spindle dynamics and proper chromosome segregation. Here we show that the effect of phosphorylation of the mouse CKAP during cell cycle on the centrosome biogenesis. Depleting the CKAP2 in NIH 3T3 induced the centrosome amplification and fragmentations leading to abnormal chromosome segregation, which was rescued by ectopic expression of wild type CKAP2. Phosphorylation is one of the key mechanisms which regulate centrosome

biogenesis and spindle assembly. We have hypothesized that phosphorylation status of the CKAP2 may be related with centrosome biogenesis. In order to explore the function of the phosphorylated CKAP2, we introduced phospho-deficient mutation at T603 which were known to be phosphorylated in human CKAP2 by Cdk1-cyclin B1, respectively. The expression of phosphorylation deficient mutant forms of mouse CKAP2, T603A could not restore the centrosomal abnormalities of CKAP2 depleted NIH 3T3 cells. These results indicates that phosphorylation of the CKAP2 is critical for both centrosome biogenesis and bipolar spindle formation. Further studies are required to elucidate the mechanisms maintaining centrosome integrity by mitosis specific phosphorylation of the CKAP2.

P173

Board Number: B281

WDR62 a critical target for the Aurora-A overexpression phenotype?.

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The Aurora-A serine/threonine kinase is frequently overexpressed in many epithelial tumors, cancers of solid organs and hematological malignancies. It has been shown that overexpression of Aurora-A is implicated in causing or maintaining the malignant phenotype and resistance to taxanes, which are effective anticancerous drugs and routinely used for the treatment of many cancers. At the physiological level Aurora-A regulates many aspects of mitosis, such as mitotic entry, bipolar formation of the mitotic spindle and centrosome regulation, including centrosome separation and centrosome maturation. However, very little is known about the molecular consequences of Aurora-A overexpression and how the overexpression is linked to tumorigenesis. In the current hypothesis Aurora-A overexpression leads to a failure in the spindle assembly checkpoint in a kinase-dependent manner, which would result in chromosome segregation errors with cytokinesis failure and chromosomally unstable tetraploid cells (Anand et al., 2003). Surprisingly, we find that Aurora-A disrupts mitotic progression in a kinase-independent way. And second, we show that exogenous Aurora-A does not impair the spindle checkpoint. It is rather that Aurora-A overexpression leads to spindle orientation defects and causes the formation of DNA bridges in anaphase. These DNA bridges are known to impair cytokinesis, which in turn could result in tetraploid cells. Searching for the causes for these mitotic defects, we set out to identify the proteins that are titrated out by Aurora-A overexpression with mass spectrometry. We found WDR62 as an interaction partner, which has been recently reported as an Aurora-A activator protein (Chen et al., 2014). Mutations in this centrosomal protein lead to microcephaly in human patients (Bilgüvar et al., 2010; Nicholas et al., 2010; Yu et al., 2010). Here, we show that the abundance of WDR62 at centrosomes is reduced in Aurora-A overexpressing cells and that depletion of WDR62 gives also rise to spindle orientation defects and DNA bridges in anaphase. Rescue experiments show that co-expression of WDR62 and Aurora-A rescues the Aurora-A overexpression phenotype (DNA bridges and spindle orientation defects). Therefore we postulate that overexpression of Aurora-A

reduces WDR62 amounts in HeLa cells, which in turn leads to cell divisions with DNA bridges that disturb cytokinesis.

P174

Board Number: B282

Localization determinants in the Mps1 amino terminus distinguish centrosomal and kinetochore targeting.

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Cells have evolved elaborate mechanisms to ensure the faithful segregation of chromosomes. The centrosome, which consists of two microtubule-based centrioles, must be precisely duplicated only once in S-phase to become the poles in a proper bipolar mitotic spindle. The spindle assembly checkpoint (SAC) serves to block the progression of mitosis until all chromosomes are properly attached to spindle microtubules from both poles. Failure to regulate either of these processes has the potential to cause aneuploidy, which is a hallmark of many cancers. The kinase Mps1 has previously been shown to regulate both the duplication of centrioles in S-phase and the SAC in mitosis. This implies that different localization prompts in Mps1 govern its localization throughout the cell cycle. Here we show that separate elements in the Mps1 N-terminus, which includes the known domains N-terminal extension (NTE), tetratricopeptide repeat (TPR), and centrosome localization domain (CLD), govern localization to either the kinetochore or the centrosome. Domains that have previously been shown to be responsible for kinetochore targeting were found to be dispensable for centrosomal localization and function. A separate motif is shown to be necessary for centrosomal targeting and the interaction with the centrosomal protein voltage dependent anion channel 3 (VDAC3), which has previously been shown to recruit Mps1 to the centrosome. Deletion of this element resulted in a version of Mps1 that maintains kinetochore localization and SAC function, while failing to elicit centriole reduplication. This data is consistent with a separation of function for the kinase based on separate elements of an N-terminal localization determinant.

P175**Board Number: B283****Differential inhibition of centrosome amplification in 2-CEES-treated Saos-2 cells by antioxidants glutathione and Trolox.**R.A. Bennett¹, E. Behrens², A. Zinn³, C. Duncheon², T.J. Lamkin⁴;¹Department of Biology, University of Southern Indiana, Evansville, IN, ²University of Southern Indiana, Evansville, IN, ³Department of Biological Science, University of Toledo, Toledo, OH, ⁴Molecular Signatures Section - Molecular Genetics, Air Force Research Laboratory, Wright-Patterson AFB, OH

Mustard gas (MG) is a chemical weapon that was first used in World War I (Duchovic and Vilensky, 2007). In recent times, its mortality and morbidity combined with its simple formulation have raised concerns over its potential use by terrorist organizations and unstable governments. MG is a powerful vesicant and alkylating agent that causes DNA monoadducts and crosslinks to form (Fidder et al, 1994). Acute exposure to MG results in painful blisters and respiratory distress, whereas chronic, low-level exposure to MG, such as that which is found in facilities that manufacture it, results in higher incidences of cancer in those exposed (Smith et al, 1995; Easton et al, 1988). Although the exact mechanism of carcinogenesis is not known, it is thought that MG induces tumor formation through its ability to induce oxidative stress and DNA damage. In addition to nucleic acids as targets of MG toxicity, several proteins have been shown to either be targets of MG-induced damage or mediate its toxicity. Among these proteins are those that have been shown to regulate centrosome biology (e.g. p53, poly(ADP-ribose) polymerase, and NFκB (Ruff and Dillman, 2010). Additionally, DNA damage and oxidative stress have been shown to induce centrosome amplification (i.e. more than 2 centrosomes per cell) as well (Dodson et al, 2004; Chae et al, 2005). Centrosomes direct the segregation of chromosomes during mitosis through the formation of the bipolar mitotic spindle. Cells with amplified centrosomes during mitosis can segregate their chromosomes unequally, resulting in chromosome instability (CIN), a common phenotype of cancer cells (Fukasawa, 2005). In our studies, we utilized the widely accepted, less-hazardous surrogate of MG called 2-chloroethyl ethylsulfide (2-CEES) to determine whether or not it could induce centrosome amplification in cells and subsequent CIN. We have previously shown that 5% and 1% of untreated Saos-2 and NIH3T3 cells, respectively, exhibit centrosome amplification, while 35% and 32% of cells, respectively, treated with 250 μM 2-CEES exhibit centrosome amplification. Cells with centrosome amplification also show an increase in CIN as measured by an increase in aneuploidy in 2-CEES-treated cells (Bennett et al, 2014). Here, we show that glutathione, a scavenger of 2-CEES molecules and reactive oxygen species (ROS), reduces the percent of cells with amplified centrosomes in 2-CEES-treated Saos-2 cells from 52% to 12.5%, while treatment with the antioxidant Trolox, an inhibitor of ROS, reduces centrosome amplification in 2-CEES-treated Saos-2 cells from 37% to 24%. These data indicate that 2-CEES can induce centrosome amplification and CIN in cells and that it may do so through both its ROS-inducing and DNA damaging capacities.

P176**Board Number: B284****Understanding how aneuploidy and multipolar mitosis are formed after chrysotile and vincristine treatments.**B.A. Cortez¹, P.R. Teixeira², S.D. Redick³, S. Doxsey⁴, G. . Machado-Santelli²;¹Depto Genetica e Biologia Evolutiva, University of São Paulo, São Paulo, Brazil, ²Department of Cell and Developmental Biology, University of Sao Paulo, Sao Paulo, Brazil, ³Univ Massachusetts Med Sch, Worcester, MA, ⁴Univ Massachusetts Med Sch, Worcester, MA

Aneuploidy is a feature of solid tumors. Aneuploid cells result from errors during mitosis, such as centrosome amplification, multipolar mitosis and cytokinesis abnormalities. The capability of aneuploidy to promote and to suppress tumorigenesis has driven efforts to characterize mitotic errors that form viable and not viable aneuploid cells. We have previously shown that chrysotile, an asbestos fiber, and vincristine, a chemotherapeutic agent, are able to induce aneuploidy and multipolar mitosis. Now we directed our focus to discover possible mechanisms involved in aneuploid cell formation after these treatments. Herein we evaluated centrosome morphology, chromosome number, location of proteins required to mitosis, and origins and fates of multipolar mitosis and multinucleated cells after chrysotile and vincristine treatment. The experiments were performed using human normal epithelial cells (RPE) and human lung cancer cells (HK2). Chrysotile fibers, in normal and cancer cells, led to mislocalization of proteins involved in intercellular bridge establishment and abscission, such as Septin 9, Anillin, Aurora B and Alix. Cytokinesis regression was observed in almost 20% of cytokinesis in the cultures after fiber treatment for 24 h, resulting in tetraploid multinucleated cells. These cells were able to enter cell cycle, giving rise to multipolar mitosis and aneuploid cells in cancer and normal lineages. Vincristine treatment led to specific and common responses in normal and cancer cells. During metaphase arrest pericentrosomal matrix was fragmented but no centriole reduplication was observed, and arrested cells could be conducted to mitotic slippage in both lineages, generating multinucleated tetraploid cells. Both cell lineages showed during recovery periods increased centriole numbers and abundant pericentriolar matrix. However, normal tetraploid cells could not progress through cell cycle and neither form multipolar mitosis, while cancer tetraploid cells showed Aurora A overexpression, centrosome abnormalities, multipolar mitosis and high levels of aneuploidy. The results showed that HK2 cancer cells could proliferate even after several mitotic errors induced by chrysotile and vincristine, while RPE normal cells could only overcome errors induced by chrysotile treatment. These observations suggest that normal cells do not display protection mechanisms against aneuploid cell formation induced by chrysotile.

Spindle Assembly 1

P177

Board Number: B285

Feedback and Spatial Organization of the Ran Pathway in Mitosis.

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During cell division, the GTPase Ran forms gradients around chromosomes. Current models of spindle assembly assume that the size of the spindle is determined by the length of these gradients. We used a new multipoint fluorescence fluctuation spectroscopy technique to study the Ran pathway in mitosis. We found that the distribution of components of the Ran pathway which regulate microtubule behaviors is determined by their interactions with microtubules, producing a feedback in which microtubule nucleators are localized by the microtubules whose formation they stimulate. Our results indicate that gradients in Ran act to trigger spindle assembly around chromosomes, but do not produce the length scales of spindle structure. Rather, the size of the spindle results from the feedback process itself, leading to robust spindle self-organization.

P178

Board Number: B286

Spatio-temporal Model for Silencing of the Mitotic Spindle Assembly Checkpoint.

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The spindle assembly checkpoint arrests mitotic progression until each kinetochore secures a stable attachment to the spindle. Despite fluctuating noise, this checkpoint remains robust and remarkably sensitive to even a single unattached kinetochore among many attached kinetochores; moreover, the checkpoint is silenced only after the final kinetochore-spindle attachment. Experimental observations showed that checkpoint components stream from attached kinetochores along microtubules toward spindle poles. Here, we incorporate this streaming behavior into a theoretical model that accounts for the robustness of checkpoint silencing. Poleward streams are integrated at spindle poles, but are diverted by any unattached kinetochore; consequently, accumulation of checkpoint components at spindle poles increases markedly only when every kinetochore is properly attached. This step-change robustly triggers checkpoint silencing after, and only after, the final kinetochore-spindle attachment. Our model offers a conceptual framework that highlights the role of spatiotemporal regulation in mitotic spindle checkpoint signaling and fidelity of chromosome segregation.

P179**Board Number: B287****A new role for the endocytic pathway during spindle pole formation and mitosis.**H.A. Hehnly^{1,2}, S. Doxsey³, J. Scott⁴;¹Univ Massachusetts, Worcester, MA, ²Department of Pharmacology, University of Washington Medical School, Seattle, WA, ³PMM, Univ Massachusetts Med Sch, Worcester, MA, ⁴Department of Pharmacology, University of Washington Medical School, HHMI, Seattle, WA

Rab11-GTPase-containing endosomes were recently implicated in spindle pole assembly and function, and in spindle orientation (Hehnly and Doxsey, 2014). However, whether their role in spindle assembly is due to a direct role in their spindle maturation was not characterized. During the cell cycle, specifically at growth phase to mitotic entry, the duplicated centrosomes mature into mitotic spindle poles to ultimately set up a microtubule-based spindle to separate duplicated genetic material into a mother and daughter cell. We examined Rab11-endosome tracks towards the centrosome/spindle pole in interphase cells compared to prophase cells. Surprisingly, we found that an increased number of vesicles transported at an elevated rate to spindle poles during prophase when compared to interphase. When we took a closer look, we observed that the Rab11 effector, FIP3, and Rab11-endosomes had a burst of recruitment to a single maturing spindle pole late in G2. This spindle was then characterized to be the oldest spindle pole that contained a 2-fold increase in mother centriole localized proteins (e.g. cenexin, centriolin) compared to the other. Based on our previous observation that Rab11 is localized to the appendages of the mother centriole and the appendage proteins, cenexin and centriolin, can mediate Rab11-endosomes organization and association with the centriole, we decided to examine whether cenexin- or centriolin-depletion disrupted recruitment of endosomes during spindle maturation. Interestingly, recruitment wasn't inhibited, but the preferential recruitment to the oldest spindle pole was diminished where both spindle poles now recruit similar ratios of Rab11-endosomes. Cenexin and centriolin-depleted cells assembled γ -tubulin to the maturing spindle pole, but there was no longer an asymmetric distribution. These cells, like Rab11-depleted cells, display asymmetric spindle orientation during mitosis. One possibility is that asymmetric protein recruitment to the oldest spindle pole is required to ensure division symmetry, and under conditions of Rab11-depletion or loss of mother centriole components spindle asymmetry becomes perturbed.

P180**Board Number: B288****The Nucleoporin ALADIN is Essential for Proper Mitotic Spindle Formation.**S. Carvalhal¹, K. Koehler², A. Huebner², E.R. Griffis¹;¹Centre for Gene Regulation and Expression, University of Dundee, Dundee, Scotland, ²Department of Pediatrics, Technische Universität Dresden, Dresden, Germany

In an RNAi screen for genes essential for mitotic progression, we have identified the nucleoporin ALADIN as a factor that is essential for timely chromosome alignment and spindle morphology. When ALADIN is depleted, we see a delay in chromosome alignment, hyperstretching of paired kinetochores, and destabilized K-fibres. When we examined the molecular nature of these defects, we observed that the protein NuMA retracts from its localization on spindle poles and concentrates on spindles. Conversely, we find that after ALADIN depletion, Aurora A kinase becomes less focused at centrosomes (where the active form is reduced by 40% compared to control cells) and spreads along spindle microtubules. We find that the relocalization of NuMA seen after ALADIN depletion can be phenocopied by inhibiting Aurora A; however, the localizations of other Aurora A substrates such as Eg5 and TACC3 are not perturbed by ALADIN depletion, suggesting that the effects we see are specific to a subset of Aurora A targets. We find that ALADIN, which normally enriches near spindle poles, can be recruited to centrosomes and can bind to Aurora A after this kinase is inhibited by MLN8237. We currently favor a model in which ALADIN scavenges inactive Aurora A to promote its reactivation and maintain a high concentration of this protein near spindle poles where it can phosphorylate substrates like NuMA. Curiously, ALADIN was first identified as a gene that is mutated in patients who have triple A syndrome, which impairs lacrimal, adrenal, and neural functioning. We have investigated the localization of mutant forms of ALADIN in mitosis, and have seen that some show a gain of function localization on kinetochores, while the expression of others inhibit chromosome alignment. We are currently testing whether these mutant forms are still able to interact with Aurora A, and we are beginning to use IPS cells to explore the possibility that mitotic defects underlie some of the symptoms of triple A syndrome.

P181**Board Number: B289****RanBP1 governs spindle assembly by defining mitotic Ran-GTP production.**M. Zhang¹, A. Arnaoutov¹, M. Dasso¹;¹National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD

Proper spindle formation requires a chromatin-based gradient of the small GTPase Ran, with high RanGTP levels in the vicinity of mitotic chromosomes. This gradient is established through the activity of Ran's nucleotide exchange factor (RCC1), which concentrates on chromatin. RanBP1 is a co-activator of Ran's GTPase activating protein (RanGAP1). It has been known for nearly two decades that RanBP1 also forms a stable heterotrimeric complex with RCC1 and Ran (RRR complex), which inhibits RCC1's RanGEF activity. The function of the RRR complex has remained mysterious, however, because RanBP1 is

physically separated from RCC1 during interphase. We found that the RRR complex formed readily in M-phase *Xenopus* egg extracts (CSF-XEEs). RCC1 binding to chromatin and RRR complex assembly were mutually exclusive, so that promoting RRR complex formation through the addition of recombinant RanBP1 sequestered RCC1 away from chromatin. Consistent with earlier reports, RRR complex assembly inhibited RCC1's RanGEF activity. Together, these findings suggest that the RRR complex plays a key mitotic role in determining the partitioning of RCC1 between its active chromatin-bound and inactive soluble states, thereby setting both the location and magnitude of mitotic Ran-GTP production. Notably, RCC1's association to mitotic chromatin is dynamic, and there are particularly large changes during the metaphase-to-anaphase window. However, the timing of reported RCC1 modifications suggests that they do not cause such changes. We found that RanBP1 is phosphorylated during anaphase, and that this modification disrupts the RRR complex. Further analysis showed that RanBP1 phosphorylation drove increased RCC1 binding to chromatin in cycling XEE, and thereby indirectly enhanced anaphase Ran-GTP production. This modification may also contribute to Ran pathway function in early interphase, because elevated RCC1 on anaphase chromatin should provide high levels of Ran-GTP to facilitate nuclear re-assembly. Finally, separation of RCC1 and RanBP1 after RRR complex dissociation allows RCC1 sequestration to re-forming nuclei while excluding RanBP1 into the early interphase cytosol. In summary, we have documented a novel role of the RanBP1 protein in controlling the localization and activity of Ran's nucleotide exchange factor, RCC1. We have shown that phosphorylation of RanBP1 during anaphase drives changes in RCC1 dynamics and allows increased Ran-GTP production. These findings resolve important and long-standing questions within the Ran field regarding the function of the RanBP1/Ran/RCC1 complex and its dynamics.

P182

Board Number: B290

The budding yeast Polo-like kinase Cdc5 is spatially regulated for timely mitotic exit and adaptation to DNA damage.

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Polo-like kinases regulate many events including mitotic entry, chromosome segregation, mitotic exit, cytokinesis, and adaptation to DNA damage; however, how Polo activity is regulated remains poorly understood. The budding yeast Polo-like kinase Cdc5 is regulated spatially during the cell cycle. Cdc5 accumulates in the nucleus upon mitotic entry, consistent with the fact that many known Cdc5 substrates are nuclear. Interestingly, in anaphase, we found that Cdc5 translocates from the nucleus to the cytoplasm. We took genetic and biochemical approaches to show that this nuclear-cytoplasmic translocation of Cdc5 in anaphase is required for activation of the Mitotic Exit Network (MEN). We found that the Cdc14 phosphatase, a downstream target of MEN, is required for this nuclear export of Cdc5. This finding suggests the existence of a positive feedback loop between Cdc5 and Cdc14 to regulate timely mitotic exit. Cdc5 also accumulates in the nucleus after DNA damage checkpoint-induced G2/M

arrest. However, by restricting Cdc5 localization either to the nucleus or cytoplasm, we demonstrated the requirement of both nuclear and cytoplasmic pools of Cdc5 for adaptation to DNA damage. Collectively, our data demonstrate that spatial regulation of Cdc5 is required for timely mitotic exit and adaptation to DNA damage.

P183

Board Number: B291

Novel mechanisms of APC regulation in mouse oocyte meiosis.

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The anaphase-promoting complex (APC) is an E3 ubiquitin ligase responsible for regulated destruction of substrates at specific stages of the cell cycle. Two APC co-activators, Cdc20 and Cdh1, mediate the timing and selectivity of substrates recognition. Cdc20 recognizes substrates containing the D-box motif only, while Cdh1-bound APC mediates the ubiquitination of proteins with either D- or KEN-box motifs. Progression throughout meiosis in oocytes utilizes the same molecular players although the start-stop nature of the female meiosis invokes additional levels of regulation. Recently, we reported that cyclin-dependent kinase 1 (Cdk1) and mitogen-activated protein kinase (MAPK) play compensatory roles in regulating the APC by suppressing its activity early in prometaphase I, thereby allowing accumulation of the APC substrates essential for meiosis I. In this study we have investigated which of the APC co-activators is responsible for this APC activity. Remarkably, we find that depletion of either Cdc20 or Cdh1 failed to prevent APC activity and that depletion of both co-activators led to increased destruction of the APC substrates. Similarly, persistent destruction of the APC substrate, securin, in Cdh1 and Cdc20-depleted prophase I-arrested oocytes points to a second cell cycle stage in which APC activity can be detected even when the known co-activators are depleted. Incomplete depletion of the co-activators seems an unlikely explanation for these findings. Western blotting reveals that the proteins are efficiently depleted in both cases and functional studies show that depletion has the predicted effects on meiotic cell cycle progression. Furthermore, the fact that co-depletion leads to an increase in APC activity, points to novel additional means of APC activation in mouse oocytes. The APC activity revealed in this study targets D-box substrates only. This observation provides further support that Cdh1 is not active, as it would also be expected to recruit KEN-box containing substrates to the APC. However, the observations are also not consistent with Cdc20-mediated APC activation given that in both prometaphase and prophase I Cdc20 depletion is without effect, Cdk1 activity is required for Cdc20 function and there is no evidence that Cdc20 can stimulate the APC during the prophase I-arrest. All these findings suggest that the instability of the D-box substrates revealed in prometaphase I and also during the prophase I-arrest might well be due to activation of the APC independently of its two major co-activators.

P184**Board Number: B292****Multiple Pathways for Spindle Assembly in Mouse Oocytes.**L. Bury¹, P.A. Coelho¹, M. Zernicka-Goetz², D. Glover¹;¹Department of Genetics, University of Cambridge, Cambridge, United Kingdom, ²Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge, United Kingdom

Mammalian oocytes are naturally acentriolar, and assemble their spindles by self-organization of cytoplasmic microtubule organizing centers (MTOCs) that congress at the center of the oocyte at the time of nuclear envelope breakdown. Although Ran-GTP contributes to the generation of sufficient microtubule density, it appears that bipolar spindles are still able to form in the absence of a stable Ran-GTP gradient, implying the existence of other factors that might support spindle assembly in the maturing oocyte. A recent study investigating acentriolar spindle formation in the early mouse embryo has discovered that centriolar proteins, namely Plk4 and Cep152, which are present despite the absence of centrosomes, are crucial for microtubule nucleation and spindle assembly. However, the mechanism of acentrosomal spindle formation in the mouse oocyte is thus far unknown. It has been hypothesized that the long duration of metaphase (6-8 hours) would allow time for multiple spindle assembly factors to form a robust microtubule network, even in the absence of a Ran-GTP gradient. On the other hand, the fact that enucleation of oocytes abrogates bipolar spindle formation, has supported the idea that indeed other nuclear factors might exist that are responsible for regulating microtubule nucleation. Consistent with studies in *Xenopus* egg extracts that point to an important role of Aurora A in chromosomal MT nucleation, we find using small molecule inhibitors that Aurora A but not Auroras B/C contribute to bipolar spindle assembly in meiosis I. Using a combination of pathway-specific inhibitors and dominant negative constructs, we find that Plk4 also contributes to this process. These two protein kinases appear to act in addition to Ran-GTP to locally stabilize microtubules surrounding chromatin, and synergism between these pathways is required to build the bipolar meiotic spindle. Interestingly, these proteins and several of their partners are concentrated in the nucleus and accumulate around chromatin at the time of the initial MT nucleation following nuclear envelope breakdown. Inhibition of any of these pathways strongly reduces the early stages of MT nucleation, but does not interfere with the actin-dependent migration of chromatin to the oocyte cortex. Eventually, cytoplasmic MTOCs nucleate small aster-like spindles and meiotic exit is able to occur.

P185**Board Number: B293****Spindle Matrix Formation is Required for Cell Cycle Progression.**J. Johansen¹, C. Yao¹, C. Wang¹, Y. Li¹, J. Girton¹, K.M. Johansen¹;¹Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA

A paramount issue in mitosis is the synchronization of events required for cell cycle progression, and although many previous studies have identified critical regulatory factors that act to coordinate the

myriad activities involved in setting up a spindle and organizing chromosomes upon it, how these diffusible regulatory factors are spatially organized and confined to the spindle region in the absence of a diffusion barrier after nuclear envelope breakdown (NEB) is not well understood. However, in *Drosophila* we have identified four nuclear proteins, Skeletor, Chromator, Megator, and EAST from two different nuclear compartments that interact with each other and that redistribute during prophase to form a dynamic, gel-like spindle matrix that embeds the microtubule spindle apparatus (MBoC 23:3532, 2012). This matrix exists independently of microtubules and the NE and specific interactions between spindle matrix molecules are necessary for complex formation and cohesion. In order to address how the spindle matrix interacts with cell cycle components we have taken a live imaging approach to determine the relative timing of localization and cross-interactions of these proteins. Previously, we have shown that the spindle matrix protein Megator and its human homolog Tpr have an evolutionarily conserved function as spatial regulators of the spindle assembly checkpoint proteins Mad2 and Mps1. Here we show that a number of key cell cycle proteins such as Cyclin B, Polo, Ran, 14-3-3 and Endos also are co-localized at enriched levels within the spindle matrix during mitosis and that this localization is independent of microtubules. Furthermore, prevention of spindle matrix formation by injection of a function blocking antibody to the spindle matrix protein Chromator results in cell cycle arrest prior to NEB phenocopying the triple RNAi knockdown of Cyclins A, B, and B3 (McClelland and O'Farrell, Curr. Biol. 18:245, 2008) Interestingly, in such embryos the dynamic relocation of Polo and Cyclin B to the nuclear rim and kinetochores is abrogated and Polo is not imported into the nucleus. This is in contrast to colchicine-arrested embryos where the wild-type dynamics of these proteins are maintained. Furthermore, we show that the spindle matrix prevents Pdi-GFP-marked vesicular membranes from entering the nuclear space after NEB although they are permeable to microtubules. These studies promise to provide a mechanistic framework for understanding how cell cycle factors are physically confined and organized in the spindle region in organisms with open or semi-open mitosis, allowing for spatial and temporal integration of signaling events leading to mitotic progression and chromosome segregation.

P186

Board Number: B294

Plk4 regulation of acentriolar MTOC architecture is essential for meiotic division of mouse oocytes.

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Spindles of most animal cells are assembled from canonical centrosomes, build up of two centrioles and their surrounding pericentriolar material. However, most oocytes lose their centrioles during their growth in the ovary yet do assemble functional meiotic spindles able to segregate chromosomes. In fully-grown mouse oocytes, acentriolar MTOCs (aMTOCs, MicroTubule Organizing Centers) gather as large pericentriolar material foci around the nuclear envelope in prophase I of meiosis. At meiosis resumption, these foci are rapidly stretched around the nuclear envelope, fragmenting into smaller

pieces in a dynein- and MT-dependent manner (Łuksza 2013). We address here the importance of such timely reorganization of aMTOCs. We show that Plk4, a major kinase in centriole duplication, plays an essential role in aMTOCs organization. First we show that endogenous Plk4 protein is present on aMTOCs in the mouse oocyte. Second, transgenic overexpression of Plk4 specifically during oocyte growth impacts the morphology of aMTOCs. Indeed it induces their precocious fragmentation in prophase I. This in turn has major consequences; increasing their MT-nucleating capacity and promoting early meiotic spindle bipolarization as well as assembly of larger spindles. Such robust spindles contain abnormal levels of incorrect bivalent attachments, which delay SAC inactivation impeding on the transition to anaphase I. These defects are not due to increased levels of Plk4 activity during meiotic divisions, since the injection of cRNAs encoding for Plk4 into fully grown oocytes does not perturb meiosis I progression nor affects aMTOCs fragmentation. We describe here a novel function for Plk4, namely in controlling pericentriolar material organization in the absence of centriole, a function that is essential for proper progression into meiosis I of mouse oocytes. Furthermore we show here that over-fragmented aMTOCs compromise Kinetochore-MTs attachments and chromosome congression during female meiosis.

Ref : Łuksza M., Queguiner I., Verlhac M.-H.* and Brunet S.* 2013. Rebuilding MTOCs upon centriole loss during mouse oogenesis. *Dev Biol*, 382: 48-56 (* : co-senior authors)

P187

Board Number: B295

The Relationship Between Localization and Function of the Chromosomal Passenger Complex in *Drosophila melanogaster* Oocytes.

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Accurate segregation of chromosomes during meiosis is required to preserve the correct amount and distribution of genetic material in eggs and sperm. Errors in chromosome segregation are the leading cause of miscarriages and also lead to genetic diseases such as Down syndrome. In female meiosis of many animals, spindle assembly occurs without centrosomes, the microtubule organizing centers of the cell. In *Drosophila* oocytes, the chromosomal passenger complex (CPC), which consists of INCENP, Aurora B kinase, Survivin, and Borealin, is required for chromosome-based spindle formation and bi-orientation of the centromeres. In addition, the CPC promotes the localization of additional spindle assembly factors, supports spindle maintenance, and participates in kinetochore assembly. How the CPC functions in all of these processes is not known, but we hypothesize that the localization of the CPC is important. At metaphase in oocytes, the CPC localizes with central spindle microtubules in a ring around the chromosomes and may be transiently at centromeres. One possibility is that the CPC starts on centromeres and then moves onto the central spindle. Alternatively, there may be two separate populations of CPC, one that localizes to centromeres and a separate population that localizes to microtubules. By creating RNAi-resistant mutant INCENP transgenes that will manipulate the localization of the CPC, we will test whether centromere-localized and/or central spindle-localized CPC

provide the functions of the CPC in oocytes. We will force the CPC to localize to the centromeres, kinetochores, chromatin and microtubules. Conversely, we will prevent CPC localization to the centromeres and microtubules. It is possible that CPC localization to chromatin or centromeres is not required for bipolar spindle formation and that when the CPC is unable to localize to centromeres, it will have only bi-orientation defects. However, if the CPC must first localize to centromeres before it can transition to the central spindle, this mutant may fail to assemble a spindle. Observation of the effect on spindle assembly and bi-orientation by these mutants will elucidate the CPC's mechanism in meiosis.

P188

Board Number: B300

PP1 antagonises Aurora B in *Drosophila* oocytes for chromosome structure, cohesion and kinetochore localisation.

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Reversible protein phosphorylation allows cells to direct progression through cell cycle. The significance of protein kinases in cell cycle regulation and progression has been well established. In *Drosophila* female germline Aurora B kinase, a member of the chromosomal passenger complex (CPC), is required for meiotic spindle assembly and chromosome segregation. We have also seen that if an Aurora B inhibitor is added after spindle assembly is complete, the spindle completely disintegrates and kinetochore proteins fail to localise. This indicates that continual phosphorylation activity is required due to either the presence of multiple phosphatases or degradation. Hence the protein phosphatases may play equally important reciprocal roles in cell cycle regulation and the processes involved. We have looked at the role of the serine/threonine protein phosphatase 1 (PP1) in female meiotic chromosome segregation and spindle assembly. In PP1 depleted oocytes we observe a gross disorganization of spindle microtubules and the length and shape of these spindles resemble prometaphase spindles suggesting a defect in progression to metaphase. In addition the karyosome, a structure into which all the chromosomes are compacted, is broken into several pieces with a loss of sister-centromere cohesion. This is surprising since PP2A is usually required for maintaining cohesion in mitotic cells. Kinetochore proteins like Spc105R however are unaffected by loss of PP1. We have also found that some but not all of these phenotypes are due to phosphorylation by Aurora B. The karyosome defect and loss of cohesion in oocytes lacking PP1 is rescued by Aurora B inhibition. In addition the loss of kinetochore protein Spc105R at centromeres upon inhibition of Aurora B is also rescued when PP1 is absent. These results suggest that PP1 may antagonize Aurora B for maintaining cohesion, karyosome integrity and kinetochore protein localization. However, the complete loss of the meiotic spindle caused by an Aurora B inhibitor is not restored by loss of PP1. Thus, other phosphatases may negatively regulate spindle assembly and/or Aurora B-dependent phosphorylation is required to maintain constant incorporation of spindle associated proteins throughout meiosis.

P189**Board Number: B301****Three-dimensional reconstruction of female meiosis in *C. elegans*.**I. Lantzsch¹, M. Srayko², S. Prohaska³, T. Müller-Reichert¹;¹Medical Faculty "Carl Gustav Carus", TU Dresden, Dresden, Germany, ²Univ Alberta, Edmonton, AB,³Zuse Institute, Berlin, Germany

In meiosis, haploid gametes are produced from a diploid progenitor cell and this process of genome haploidization differs between oogenesis and spermatogenesis. Similar to many other species, including *Drosophila*, *Xenopus*, mice and humans, the female meiotic spindles in *C. elegans* are anastral and acentrosomal [1,2]. At early metaphase, the microtubules form an elongated bipolar spindle, which is about 10 µm in length. The metaphase spindles then shorten to roughly 3-4 µm and adopt a barrel-like shape. During late anaphase, microtubules are only observed in the interzone between the separating chromosomes/chromatids. The mechanism of this reorganization of the microtubules during the metaphase-to-anaphase transition is not understood [3]. Here, we show three-dimensional reconstructions of meiotic spindles at different stages. The 3D models were obtained by correlative light and electron tomography. Our anaphase I model shows about 4000 microtubules in between the chromosomes. Currently, we are analyzing the average length and the length distribution of microtubules to test models of chromosome segregation. In addition, we are investigating, whether microtubule assembly in this anaphase 'inside-out' spindle nucleation is taking place close to the surface of the chromosomes. Parallel to this wild-type characterization of the female meiotic spindles, we are characterizing candidate genes involved in various aspects of spindle assembly. One candidate protein for 3D reconstruction is the microtubule depolymerising kinesin-13 member, KLP-7/MCAK.

1. Manandhar et al. (2005). Biol Reprod. 72, 2-13.
2. Müller-Reichert et al. (2010). Cell Mol Life Sci. 67, 2195-2213.
3. Dumont et al. (2010). Nature Cell Biology. 12, 894-901.

P190**Board Number: B302****Feedback control of chromosome separation by a midzone Aurora B gradient.**O. Afonso¹, I. Matos^{1,2}, A. Pereira¹, P. Aguiar^{1,3}, H. Maiato^{4,5};

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Accurate chromosome segregation during mitosis requires the physical separation of sister chromatids prior to nuclear envelope reassembly (NER). However, how these two processes are coordinated

remains unknown. Using live-cell imaging, RNAi, pharmacological inhibitions and laser microsurgery in *Drosophila* S2 cells, we identified a conserved feedback control mechanism that delays chromosome decondensation and NER in response to incomplete chromosome separation during anaphase. A midzone-associated Aurora B gradient was found to monitor chromosome position along the division axis and prevent premature chromosome decondensation by retaining Condensin I. PP1/PP2A phosphatases counteract this gradient to trigger chromosome decondensation and NER. Thus, the Aurora B gradient appears to mediate a surveillance mechanism that prevents chromosome decondensation and NER until effective separation of sister chromatids. This promotes the correction and re-integration of lagging chromosomes in the main nuclei prior to completion of NER.

P191

Board Number: B303

Chromosome segregation during male meiosis in *C. elegans*.

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We aim to characterize male meiosis I and II in *C. elegans*. Germ cells in the male worm mature while slowly moving from the distal to the proximal part of the gonad ^[1]. During this process undifferentiated germ cells leave the mitotic region at the distal tip of the gonad and undergo a transition to early stages of meiotic prophase I. After getting disconnected from the syncytial core (rachis), the germ cells form primary spermatocytes, while undergoing a series of cytological events during the formation of the bipolar spindles. Segregation of homologous chromosomes in meiosis I and sister chromatids in meiosis II results in four haploid spermatids from one single primary spermatocyte ^[2]. We apply both light microscopy and electron tomography to gain novel insights into the dynamics and ultrastructure of the male meiotic spindles. Using light microscopy, we image meiotic divisions within immobilized males that are fluorescently labeled with GFP::gamma-tubulin and mCherry::histone H2B. We currently determine the dynamic properties of both meiotic spindles in wild-type specimens. Furthermore, we apply laser microsurgery to manipulate the segregation of the single sex chromosome. For electron tomography, we apply high-pressure freezing followed by freeze-substitution of whole animals ^[3]. After semi-thick sectioning (300 nm serial sections) through whole worms, we aim to localize the meiotic region inside the male gonad in order to reconstruct and segment different stages of meiotic spindles in ultrastructural detail. Comparing wild-type and mutant situations ^[4, 5], our long-term goal is to integrate the data on spindle dynamics and ultrastructure to develop a mechanistic model of spindle assembly and sex chromosome segregation in *C. elegans* male meiosis.

^[1] Kimble, J. and Crittenden, S. L.; Germline proliferation and its control; 2005; WormBook. ^[2] Chu, D. S. and Shakes, D. C.; Spermatogenesis; 2013; Germ Cell Development in *C. elegans*. ^[3] Müller-Reichert, T., et al.; Cryoimmobilization and 3D visualization of *C. elegans* ultrastructure; 2003; J Microsc. ^[4] O'Connell, K. F., et al.; A genetic screen for temperature-sensitive cell-division mutants in *C. elegans*; 1998; Genetics. ^[5] Dernburg, A. F., et al.; Meiotic recombination in *C. elegans* initiates by a conserved mechanism and is dispensable for homologous chromosome synapsis; 1998; Cell.

P192**Board Number: B304****Metabolic Imaging and the Role of Cellular Energy in Cell Division.**T. Sanchez¹, D. Needleman¹;¹Harvard School of Engineering and Applied Sciences, Cambridge, MA

Metabolic defects and chromosome segregation errors co-occur in cancer, non-competent embryos resulting in failed pregnancies, and aging. It is unclear to what extent metabolic defects cause chromosome segregation errors, or chromosome segregation errors cause metabolic defects, or both are caused by other factors. To study this issue, we are investigating the relationship between mitochondrial metabolism and chromosome segregation in *C. elegans* and mouse oocytes and embryos.

We are performing quantitative metabolic imaging by measuring endogenous NADH and FAD fluorescence with fluorescence lifetime imaging microscopy (FLIM), allowing us to determine the concentrations, lifetimes, and enzyme interactions of these central metabolites. Our preliminary results show clear distinctions between metabolic states of various cell types. We are quantitatively characterizing how defined biochemical and genetic perturbations produce metabolic changes in these cells, and we will study the extent of corresponding changes in spindle morphology and dynamics, and chromosome segregation rates.

P193**Board Number: B305****Adaptability of Intracellular Structures to Variations in Cell Size.**M.C. Good¹, M.D. Vahey², D.A. Fletcher^{3,4,5}, R. Heald¹;

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Cells exist in a wide variety of shapes and sizes – from round eggs millimeters in diameter to highly elongated neurons with sub-micron extensions. Despite dramatic physical differences, growth and division of these cells is dependent on functional organelles, requiring that these intracellular structures adapt to a wide range of cell geometries. The mitotic spindle, a dynamic microtubule-based structure required for chromosome segregation, provides an important example of this flexibility. During *Xenopus* early embryo development, cell volume reduces nearly one million-fold due to division in the absence of growth, and spindle size scales with the spatial dimensions of the cell. An interesting question is how intracellular size regulation is achieved. Possible explanations include changes in cytoplasmic composition tied to a developmental program or direct coupling of spindle assembly to physical parameters such as cell volume and diameter. The difficulty of modulating cell size in embryos makes it hard to differentiate between these hypotheses, necessitating the construction of cell-like system with

controllable size. By combining droplet microfluidics and cell-free cytoplasmic extracts prepared from various stages of development, I was able to generate functional spindles and nuclei inside compartments whose diameter can be tuned from microns to millimeters, recapitulating the spindle size-scaling trend observed during *Xenopus* embryogenesis. By modulating droplet diameter and geometry, I discovered that metaphase spindle size is set by compartment volume, not shape, suggesting that limiting amounts of cytoplasmic material can restrict spindle growth. Various experimental results and a mathematical model support the hypothesis that tubulin is at least one component whose levels become limited by the cell volume decrease in early development, thereby constraining spindle length and matching it to cell size. In the future, by extending this encapsulation technology to other organelles and intracellular processes, it should be possible to uncover additional principles of size regulation and identify new pathways that respond to cell size. The significance of this research lies in the intimate connection between cell size and embryo development, and the observation that cell and organelle size are often misregulated in disease.

P194

Board Number: B306

Role of heat shock protein 70 in the assembly of mitotic spindles.

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The expression of heat shock protein 70 (HSP70) is high in many tumors. The elevated level of HSP70 negatively correlates with prognosis and efficacies of chemotherapeutics, implying a supporting role of HSP70 in tumor cell survival. Accumulated studies also demonstrated that HSP70 interacts with proteins of mitotic structures including spindle poles, microtubule motors, and kinetochores, indicating that HSP70 may involve in the regulation of assembly of mitotic structures and mitosis progression. In this study, we explored the role of HSP70 during mitosis by treating cells with a HSP70 inhibitor or transducing cells with HSP70-specific shRNA. The effects of these treatments on mitosis progression, mitotic spindle assembly, and cell viability were investigated. Our results showed that HSP70 accumulated at the spindle pole and co-localized with γ -tubulin and pericentrin during mitosis. Inhibition of HSP70 or depletion of HSP70 by shRNA transduction led to disruption of microtubule assembly, formation of abnormal mitotic spindles, and interference of mitosis progression. These results indicate that HSP70 may be required for the assembly and/or maintenance of a functional mitotic spindle.

P195**Board Number: B307****Clinically Relevant Concentrations of Taxol Cause Chromosome Missegregation Rather than Mitotic Arrest.**L.M. Zasadil¹, M. Burkard², B.A. Weaver¹;¹Cell and Regenerative Biology, Univ Wisconsin-Madison, Madison, WI, ²Department of Medicine, Univ Wisconsin-Madison, Madison, WI

Taxol, a microtubule-stabilizing drug, is commonly used to study fundamental mechanisms of mitosis. It is also used in the clinical setting as a chemotherapeutic agent to treat a variety of cancers. For the three decades that taxol has been used in humans, it has been thought to exert its antitumor effects by arresting cells in mitosis, since this is observed at concentrations typically used in cell culture. After treatment with μM or high nM concentrations of taxol, cells with an intact mitotic checkpoint arrest in mitosis, which often leads to cell death. Though not directly shown, it has been widely assumed that taxol also causes mitotic arrest in patient tumors in vivo. In a clinical study, we have measured tumor response and intratumoral drug concentration in breast tumors following taxol treatment. Our data indicate that intratumoral concentrations of taxol are lower than those traditionally attained in cell culture. Moreover, mitotic arrest is not necessary for tumor shrinkage in response to taxol therapy. However, the incidence of multipolar spindles uniformly increased in patient tumors following taxol treatment. These features are mimicked by treatment of cultured cells with clinically relevant concentrations of taxol, which vary by cell type but are in the low nM range. Unlike treatment with higher concentrations, treatment with clinically relevant doses of taxol does not cause a substantial mitotic arrest or an increase in death from interphase without passage through mitosis in the presence of the drug. Instead, cells treated with clinically relevant concentrations of taxol divide with relatively normal kinetics on multipolar spindles to form two or three daughter cells, resulting in chromosome missegregation. Timelapse imaging reveals that initially bipolar spindles evolve into multipolar spindles, which are then often partially focused before anaphase onset, to produce two-or-more-way divisions of DNA. Exposure to clinically relevant doses of taxol is sufficient to induce aneuploidy in chromosomally stable cells and causes cell death in both chromosomally stable and unstable cells. The success of taxol and its presumed mechanism of action have resulted in the development of additional drugs intended to cause mitotic arrest, including those targeting Eg5 and Plk1. These drugs have not yet shown the level of clinical success taxol has, perhaps because the antitumoral effects of taxol are due to chromosome missegregation rather than mitotic arrest. Although taxol is considered highly effective, only approximately half of all patients experience tumor shrinkage after taxol treatment. At present, there is no way to identify and selectively treat patients that will benefit from taxol therapy. These data reveal an alternate mechanism of action for taxol that may be useful in identifying mechanisms of resistance to this type of chemotherapy.

P196**Board Number: B308****An RNAi screen to identify genes required for acentrosomal spindle function in *C. elegans*.**I. Wolff¹, T.J. Mullen², C.M. Heath¹, A.C. Davis¹, M. Tran¹, S.M. Wignall¹;¹Northwestern Univ, Evanston, IL, ²Northwestern Univ, Chicago, IL

During cell division in most cell types, duplicated centrosomes nucleate microtubules and ultimately form the poles of the spindle. However, in many species, oocytes lack centrosomes and therefore spindle assembly occurs through a different mechanism. Our aim is to investigate acentrosomal spindle formation by identifying proteins that are required for proper spindle assembly and chromosome congression in *C. elegans* oocyte meiosis. We have completed a screen of 2,025 genes that were previously classified as embryonic lethal in genome-wide screens. This class includes genes known to be required for acentrosomal meiotic spindle function, such as *mei-1* and *mei-2* (regulators of microtubule stability), *aspm-1* (spindle organization) and *bub-1* (kinetochore component). We screened for defects in spindle formation and/or chromosome alignment using worms co-expressing GFP::histone and GFP::tubulin to visualize chromosomes and microtubules simultaneously. Additionally, our screening strain carried a temperature-sensitive mutation affecting the Anaphase Promoting Complex (APC) that prevents entry into anaphase I, allowing us to arrest oocytes at metaphase of meiosis I to better focus on spindle formation and chromosome alignment. Out of 2,025 genes screened, depletion of 291 led to gross defects in the worm, such as aberrant gonad morphology, sterility and defective worm growth; therefore, these could not be screened for meiotic spindle defects. Of the remaining genes, 63% were embryonic lethal under our conditions. 400 of these showed defects in spindle morphology such as monopolar spindles, multipolar spindles, disorganized spindles, as well as defects in chromosome congression. As expected, *MEI-1*, *MEI-2*, *ASPM-1* and *BUB-1* were in this class and resulted in dramatic spindle morphology defects following RNAi. In addition, a number of genes that are uncharacterized in worms were shown to cause defects in spindle morphology, some of which have putative homologs in other organisms. Initial results from our screen and preliminary characterization of our candidate genes will be reported. Future characterization of the genes identified in our screen may help us better understand the mechanisms involved in acentrosomal spindle assembly.

P197**Board Number: B309****Cenexin, a mother centriole appendage protein, is required for spindle orientation during mitosis.**H. Hung¹, H. Hehnlly^{1,2}, S. Doxsey¹;¹Univ Massachusetts Med Sch, Worcester, MA, ²Univ of Washington, Seattle, WA

Polarized epithelial cells are required for organ function, but whether this polarization is initially established during late cell cycle has not been clearly resolved. One argument for establishment during late cell cycle is that the epithelial layer can undergo constant regeneration to replace dead and/or damaged cells. During this time, it is known that appropriate orientation of the mitotic spindle relative to cell polarity cues can contribute to cell fate determination, which would thus establish tissue architecture. Another contributor to spindle orientation is the spindle pole. More specifically, one of the two spindle poles contains more mother centriole appendage proteins than the other. Based on this, we propose a model where the centrosome with elevated appendage proteins and polarity cues at the cell cortex establish spindle orientation. One cell cortex polarity complex examined, NuMA/LGN/Gai, is implicated in spindle orientation through its interaction with astral microtubules. In genetically-engineered kidney epithelial cells (MDCK) we found that cells depleted of the sub-distal appendage component, Cenexin, lead to asymmetric spindle orientation in both two- and three-dimensional (3D) cell culture systems. In contrast, depletion of the distal appendage protein, Cep164 had no effect on spindle orientation. In 3D culture, Cenexin-depleted cells induced a 2-fold increase in the number of lumina compared to control, which correlated with increased spindle misorientation. Surprisingly, an increase in cortical NuMA localization, and disorganization of a microtubule destabilizer, Kif2a, at the pole was also observed with Cenexin-depletion. Taken together we propose that Cenexin modulates spindle orientation by maintaining spindle pole localized Kif2a, which regulates microtubule organization and contributes to cortical NuMA localization.

P198**Board Number: B310****Mitotic Repertoire of Acetylated Survivin.**S.P. Wheatley¹, A.M. Ajaberi¹;¹School of Life Sciences, Univ Nottingham, Nottingham, United Kingdom

Survivin is a cancer associated protein that was originally cloned as a member of the inhibitor of apoptosis (IAP) family, and later shown to be an essential mitotic protein. The dual pro-survival functions of survivin depend on the localization of the protein within immunochemically distinct pools located in cytoplasm, mitochondria, nucleus and on the chromosomes. During mitosis, survivin operates in a complex with the chromosomal passenger proteins, aurora-B kinase, borealin and INCENP, which localises initially to the centromeres where it regulates chromosomal movements, and later to the equatorial cortex of the cell where it is required for cytokinesis. The mitotic activity of survivin is

phospho-regulated by a number of kinases including Cdk1, aurora-B and plk1. Recently, it has been shown that residue K129 in its C-terminal alpha helical coil is acetylated and that this post-translational modification affects the subcellular localisation of survivin during interphase, however, whether this acetylation event contributes to the regulation of mitosis remains unknown. In this study, we ask whether mimicking acetylation by mutating this site to an alanine alters the behaviour of during mitosis. Our data demonstrate that constitutive acetylation of this site inhibits cell proliferation and causes errors in chromosome segregation during mitosis.

P199

Board Number: B311

Microtubule dynamics are modulated by changes in cytoplasmic volume: a possible mechanism for mitotic spindle scaling.

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Spindle size is determined by mechanisms intrinsic to the spindle itself, such as motor-dependent sliding and microtubule polymerization dynamics, and by extrinsic mechanisms like cell size and cortical/cytoplasmic pulling forces. The combined action of these processes ensures constancy of spindle size and shape and a high fidelity of chromosome segregation during cell division. During early development in animals, changes in cell size that accompany rapid reductive divisions result in cells with smaller and smaller spindles, likely due to a progressive reduction in the available amounts of essential spindle components. The mechanistic link between component limitation and spindle size remains poorly understood. Recent studies suggest that changes in microtubule polymerization dynamics, specifically growth rate, could account for spindle scaling during development, with spindle length exhibiting a positive correlation with microtubule growth rates. This led us to predict that microtubule growth rates might be influenced by cell size. To investigate the relationship between microtubule dynamics and cell size, we combined microfluidics and cell-free extracts to encapsulate artificial microtubule organizing centers and sperm nuclei in discrete droplets of *X.laevis* egg extract. The extract was spiked with fluorescently labeled tubulin and mCherry-EB1, a protein which associates with the growing microtubule ends forming fluorescent comets. Microtubule dynamics we characterized by EB1+TIP tracking using ClusterTrack MATLAB software package. Our measurements indicate that microtubule growth velocities correlate with extract volume. These results suggest that volume-dependent modulation of microtubule growth rates is mechanism linking limiting cytoplasmic pools with changes in spindle size.

P200**Board Number: B312****Minus-End Directed Kinesin-14 Motors Align Anti-Parallel Microtubules to Control Metaphase Spindle Length.**

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During cell division, a microtubule-based mitotic spindle mediates the faithful segregation of duplicated chromosomes into daughter cells. Proper length control of the metaphase mitotic spindle is critical to this process, and is thought to be achieved through a mechanism in which spindle pole separation forces from plus-end directed motors are balanced by forces from minus-end directed motors that pull spindle poles together. However, in contrast to this model, metaphase mitotic spindles with inactive Kinesin-14 minus-end directed motors often have shorter spindle lengths, along with poorly aligned spindle microtubules. A mechanistic explanation for this paradox is unknown. Using computational modeling, in vitro reconstitution, live-cell fluorescence microscopy, and electron microscopy, we now find that the budding yeast Kinesin-14 molecular motor Kar3-Cik1 can efficiently align spindle microtubules along the spindle axis. This then allows plus-end directed Kinesin-5 motors to efficiently exert the outward microtubule sliding forces needed for proper spindle bipolarity.

Cytokinesis 1**P201****Board Number: B313****Investigating the role of F-BAR proteins in cytokinesis.**

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The goal of this research project is to identify the role that F-BAR proteins play during cytokinesis in *D. melanogaster* S2 cells. It was discovered that F-BAR proteins are essential for fission yeast cell division and act cooperatively as membrane-bending proteins (Martin-Garcia et al. J. Cell Sci. 2014); and recent work has shown that a *Drosophila* F-BAR protein is also required for coupling the membrane to the contractile ring (Takeda et al. Open Biology 2013). The first component of this research project is to identify if there are other F-BAR proteins involved in cytokinesis in *Drosophila* cells, and if they behave synergistically. This will be achieved by using dsRNAs to knock down six specific F-BAR containing proteins alone and in combinations. Cells treated with dsRNAs were imaged and the images were

analyzed to determine the percentage of multinucleate cells. Preliminarily, each F-BAR knockdown induced a higher percentage of multinucleate cells than untreated control cells. However, a combinatorial knockdown of two F-BARs at once was not shown to induce a stronger multinucleate phenotype.

The second component of this research project is to discover the cellular localization of the six F-BAR domain-containing proteins. The localization of these proteins will be made possible by creating GFP tagged F-BAR constructs. These constructs can be transfected into cells for fixed and live-cell imaging. When we observed GFP-F-BAR-CG8176 and mcherry-tubulin, in S2 cells spread on ConA, we discovered that the F-BAR localized in spots on the basal cell membrane radiating from the center of the cell. GFP-F-BAR-CG33094 exhibited an identical localization.

Further dsRNA knockdown experiments are required to confirm whether certain F-BAR depletions cause cytokinetic defects. Also, the matching localization of two separate F-BAR proteins suggests a shared localization among many F-BAR domain-containing proteins in S2 Cells. Further cloning and imaging is necessary to determine the localization of F-BAR proteins during the process of cytokinesis.

P202

Board Number: B314

Cdc14 Regulates Actin Ring Formation in Budding Yeast Via Dephosphorylation of Iqg1.

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In budding yeast, the IQGAP protein Iqg1 is required to recruit actin filaments to the actomyosin ring, as well as for contraction of the ring during cytokinesis. Iqg1 contains four perfect CDK consensus sites in the N-terminus, and mutation of these sites to alanine (*iqg1-4A*) leads to premature formation of the actin ring prior to anaphase onset, and prevents proper actomyosin ring contraction. Since Cdc14 dephosphorylates many CDK targets, we looked at the effects of manipulating Cdc14 levels on actomyosin ring formation. Overexpression of Cdc14 induces premature actin ring formation in nocodazole arrested cells. Using a *cdc14-1* temperature sensitive mutation to inactivate Cdc14 and overexpression of Sic1 to bypass the mitotic arrest of *cdc14-1* cells, we show that Cdc14 is required for actin ring formation. This defect can be rescued by expressing *iqg1-4A*, demonstrating that Iqg1 is the sole target of Cdc14 that regulates actin ring formation. Iqg1 is the first actomyosin ring target identified for Cdc14.

P203**Board Number: B315****Anillin proteins regulate intercellular bridge stability to maintain syncytial organization of the *C. elegans* germline.**R. Amini¹, A. St-Pierre-See², N.T. Chartier², J. Labbe³;¹Systems Biology, IRIC, Univ Montral, Montreal, QC, ²Pathology and Cell Biology, IRIC, Univ Montral, Montreal, QC, ³Institute for Research in Immunology and Cancer, Department of Pathology and Cell Biology, University of Montreal, Montreal, QC

Cells typically complete their division with cytokinesis. However, during development of certain tissues, mitotic division is followed by incomplete cytokinesis, giving rise to interconnected cells in a shared cytoplasm, or syncytium. Syncytia are a conserved feature of female and male germline of most species. Despite this, how syncytium formation is regulated remains elusive. To understand this, we studied the syncytial *C. elegans* germline wherein germ cells (GCs) remain connected via intercellular bridges. Using live-cell imaging, we found that the contractility regulators ANI-1 (Anillin), NMY-2 (myosin), UNC-59 (septin), ZEN-4 (MKLP1) and CYK-7 localize to GC intercellular bridges. ANI-2, a shorter isoform of Anillin, is enriched between the two primordial GCs from the onset of GC specification during embryogenesis and further accumulates at the intercellular bridges in the gonad. Analysis of ANI-2 localization revealed that syncytial architecture occurs progressively during larval development. Loss of ANI-2 disrupted intercellular bridge integrity and resulted in GC multinucleation, and thus severe gonad disorganization. In contrast, ANI-1 depletion partially restored syncytial organization in *ani-2* mutants, suggesting that a balance of activity between the two Anillin proteins at the intercellular bridge is required for the proper organization of the syncytial germline. We are currently investigating the mechanism by which the germline syncytium is formed to understand how controlled incomplete cytokinesis promotes formation of the syncytial *C. elegans* gonad.

P204**Board Number: B316****The role of cortical dynein during *C. elegans* female meiotic spindle rotation.**J. Flynn¹, M. Ellefson¹, K. Messina¹, P. Kuehnert¹, F. McNally¹;¹Univ California-Davis, Davis, CA

Meiosis is a specialized cell division required for sexual reproduction characterized by a single round of DNA replication followed by two rounds of chromosome segregation. The molecular machine responsible for accurately segregating chromosomes is the meiotic spindle. In female meiosis, to properly segregate chromosomes the spindle must correctly orient its pole-to-pole axis perpendicular to the cell cortex. In *C. elegans*, this perpendicular orientation is achieved through a dynein dependent process called spindle rotation. Spindle rotation has been shown to correlate with increased dynein localization on the spindle poles (Ellefson 2011, *J. Cell Biol.* 193:1229). However, in the female meiotic embryo, dynein has two distinct subcellular localizations: the spindle poles and the cell cortex. This begs

the question of whether or not cortical dynein plays a role in spindle rotation during female meiosis. To address this question, we will identify proteins required for cortical localization of dynein during meiosis then test whether these proteins are required for spindle rotation. We found that dynein recruitment to the cortex is not microtubule dependent, but is LIN-5 dependent. Other candidates that may be involved in recruiting dynein to the cortex during meiosis include proteins that are known to recruit dynein to the cortex in the *C. elegans* one cell mitotic embryo (Galpha and GPR), a protein that has been shown to recruit dynein to the cortex in an anaphase specific pathway in HeLa cells (Band4.1), and a cortical protein shown to suppress lethality induced by a conditional dynein heavy chain mutant (*efa-6*). We are currently testing whether depletion of these proteins prevents cortical dynein localization and meiotic spindle rotation.

P205

Board Number: B317

The Chromosomal Passenger Complex (CPC) couples the actin and microtubule cytoskeletons during cytokinesis.

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In animal cells, the microtubule (MT) and actin cytoskeletons are remodeled throughout cell division to facilitate chromosome segregation and cytokinesis. During anaphase, MTs organize into the spindle midzone while actin and myosin assemble into the cleavage furrow. Anaphase events – chromosome segregation, centrosome separation and cleavage furrow contraction – are closely coupled in space and time, implying that mechanisms may exist to link them. Indeed, other cellular processes that rely on both cytoskeletons, such as cell migration, use signaling-based feedback mechanisms to coordinate the activities of the actin and microtubule networks. We identified a novel pathway that stabilizes spindle midzone MTs (mMTs) at the onset of cleavage furrow ingression. Stabilization of mMTs during late anaphase depends on acto-myosin contractility, strongly suggesting that cross-talk between the actin and MT cytoskeletons occurs. We found that cells lacking CPC activity do not stabilize mMTs following cleavage furrow ingression, suggesting a novel role for the CPC in regulating MT stability during cytokinesis. The CPC is transported to mMT plus ends at the onset of anaphase by the kinesin Mklp2, and we have determined that the CPC also localizes to the cell cortex during anaphase *via* direct actin binding. Additionally, overexpression of CPC components rescues cytokinetic defects caused by Mklp2 depletion indicating there may be multiple pathways for targeting the CPC to the midzone. We propose a model in which actin binding by the CPC provides a second pathway to help recruit the complex to the cleavage furrow, and maintain its localization during anaphase and cytokinesis. We also propose that actin binding allows the CPC to mediate cross-talk between the actin and MT cytoskeletons, ultimately stabilizing mMTs following cleavage furrow ingression.

P206**Board Number: B318****Rac Antagonism of Cytokinesis in the Early Sea Urchin Embryo.**A. Ellis¹, S.P. Sepulveda¹, A. Alvarez², R. De La Rosa¹, J.H. Henson³, C.B. Shuster¹;¹Biology Department, New Mexico State University, Las Cruces, NM, ²New Mexico State University, Las Cruces, NM, ³Dickinson Coll, Carlisle, PA

z, John Henson and Charles B. Shuster Rho GTPases are critical modulators of the actin cytoskeleton, and while the role of RhoA during cytokinesis is well established, less is known about how Rac and Cdc42 contribute to cytoskeletal organization during the final stages of cell division. Neither Cdc42 nor Rac is thought to be required for cytokinesis, and several studies indicate that Rac inactivation is necessary for the completion of cytokinesis, possibly by suppressing Rac-mediated cell adhesion at the cell equator. To better understand the regulation of actin in the early embryo as well as the role of Rac during cytokinesis, we performed live cell imaging of actin dynamics during the first cell division of the sea urchin embryo. Studies to date revealed that in addition to microvilli and the contractile ring, there are other distinct populations of actin filaments that underwent dramatic changes during the first mitotic division. A thin layer of cortical actin could be detected that underwent a transient thinning upon anaphase onset, only to recover shortly before cytokinesis. Additionally, there was an explosive elaboration of cytoplasmic actin occurring just prior to the metaphase-anaphase transition, which initiated at the cell surface, and extended inward towards the cell center. Interestingly, RhoA activity was necessary for all actin structures in the egg, whereas neither Rac nor Cdc42 were required. However, expression of constitutively active Rac (Q61L) resulted in early cleavage arrest with aberrant cortical actin projections. Because there are several possible downstream pathways by which Rac may antagonize cytokinesis, well-characterized, effector-binding mutants were employed. Expression of Q61L Rac containing the PAK-binding mutation (Y40C) suppressed the aberrant cortical actin projections, but still resulted in cytokinetic failure during the first two cleavages. In contrast, expression of Q61L Rac carrying the F37A mutation, which affects Arp2/3 mediated actin polymerization, resulted in normal cleavage and actin organization. Together, these findings suggest that in non-adherent, spherical cells, Rac activity plays a direct role in antagonizing the cytokinetic apparatus, possibly through the promotion of Arp2/3-nucleated actin arrays. Current efforts are focused on verifying this notion, as well as examining whether Rac plays an active role in suppressing Rho activation during anaphase and cytokinesis.

P207**Board Number: B319****Regulation of Rho GEF Rgf3 by the arrestin Art1 in fission yeast cytokinesis.**R. Davidson¹, D. Laporte², J. Wu^{1,3};¹Department of Molecular Genetics, The Ohio State University, Columbus, OH, ²Biochemistry and Cellular Genetics, Institute of Biochemistry and Cellular Genetics, Bordeaux, France, ³Department of Molecular and Cellular Biochemistry, The Ohio State University, Columbus, OH

Rho GTPases, activated by guanine nucleotide exchange factors (GEFs), are essential regulators of polarized cell growth, cytokinesis, and many other cellular processes. However, the regulations of Rho GEFs themselves are not well understood. Rgf3 is an essential GEF for Rho1 GTPase in fission yeast cytokinesis. Here we show that Rgf3 protein levels and localization are regulated by arrestin-related protein Art1. *art1Δ* cells lyse during cell separation with a thinner and defective septum. Same as Rgf3, Art1 concentrates to the contractile ring starting at early anaphase and spreads to the septum during and after ring constriction. Moreover, Art1 localization depends on its C-terminus and Art1 is important for maintaining Rgf3 protein levels. Biochemical experiments reveal that Rgf3 C-terminus binds to Art1. Using an Rgf3 conditional mutant and with mislocalization experiments, we find that Art1 and Rgf3 are interdependent for localization to the division site. As expected, active Rho1 levels at the division site are reduced in *art1Δ* and *rgf3* mutant cells. Taken together, these data reveal a role for the arrestin-family protein Art1 in regulating the protein levels and localization of the Rho GEF Rgf3, which in turn modulates active Rho1 levels to build a division septum during cytokinesis.

P208**Board Number: B320****The putative Rho-GEF Gef3 interacts with the septin complex and regulates the GTPase Rho4 during fission yeast cytokinesis.**N. Wang¹, M. Wang¹, Y. Zhu¹, T. Grosel¹, D. Sun¹, J. Wu^{1,2};¹Department of Molecular Genetics, The Ohio State University, Columbus, OH, ²Department of Molecular and Cellular Biochemistry, The Ohio State University, Columbus, OH

Rho GTPases, activated by guanine nucleotide exchange factors (GEFs), are highly conserved molecular switches for signal transduction and regulate diverse cellular processes including cell polarization and cytokinesis. The fission yeast *Schizosaccharomyces pombe* has six Rho GTPases (Cdc42 and Rho1-Rho5) and seven Rho GEFs (Scd1, Rgf1-Rgf3, and Gef1-Gef3). The Rho GEFs for Rho2-Rho5 have not been unequivocally assigned. Gef3, the smallest Rho GEF, was barely studied. Here we show that Gef3 colocalizes with septins to a non-constricting double rings at the cell equator during division-septum formation. Gef3 physically interacts with and depends on the septin complex to localize. Purified Gef3 strongly interacts with Rho4 GTPase in vitro. Consistently, Gef3 and Rho4 are in the same genetic pathways to regulate septum formation and cell separation. Although localization of Rho4 is not affected in *gef3Δ*, the localizations of two potential Rho4 effectors, glucanases Eng1 and Agn1, are

abnormal, indicating a compromised activation of Rho4. Moreover, overexpression of active Rho4 or Eng1 rescues the cytokinesis defects of exocyst mutants and mutants containing *gef3Δ*. Together, our data suggest that the putative Rho-GEF Gef3 interacts with the septin complex and regulates GTPase Rho4 for septum formation and cell separation during cytokinesis.

P209

Board Number: B321

The RhoGAP domain in centralspindlin directly contributes to activation of RhoGEF ECT-2 during cytokinesis.

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In animal cells, the small GTPase RhoA is essential for the contractile actomyosin network that drives cytokinetic furrowing. Counterintuitively, the evolutionarily conserved protein complex, centralspindlin, which contains a subunit, CYK-4, with a Rho family GAP domain, promotes RhoA activity. The regulatory N-termini of CYK-4 and the RhoA GEF ECT-2 directly interact. Various studies have indicated that the GAP domain is important for CYK-4 function, but the underlying mechanism remains controversial.

To determine whether the catalytic activity of CYK-4 GAP domain contributes to cytokinesis, we established single-copy transgenes in *C. elegans*, and mutated the catalytic residue R459 to specifically perturb the GAP activity of CYK-4. *cyk-4^{R459A}* mutant embryos fail to complete cytokinesis. RhoA activation involves a parallel, non-essential, nematode-specific activator of ECT-2, NOP-1. *cyk-4^{R459A};nop-1* embryos exhibit extremely weak furrow ingression. Mutations in CED-10/Rac1 do not modulate the defect in *cyk-4^{R459A};nop-1* mutant embryos, demonstrating that the defect resulting from loss of catalytic activity of the GAP domain is not due to hyperactivation of CED-10/Rac1. To investigate whether furrow ingression defects result from failure to fully activate or inactivate RhoA, we examined how the defect was modulated by depletion of the primary RhoA GAP, RGA-3/4. Remarkably, depletion of RGA-3/4 allowed cytokinetic completion in both *cyk-4^{R459A}* and *cyk-4^{R459A};nop-1* mutant embryos. Binding assays revealed that CYK-4^{R459A} binds to RhoA more strongly than CYK-4. To test whether the CYK-4^{R459A} cytokinesis defect results from sequestration of RhoA, we mutated charged surface residues in CYK-4. These variants reduce RhoA binding and cause more severe RhoA activation defects than CYK-4^{R459A}. These results suggest that CYK-4 has to bind to RhoA in order to fully activate ECT-2 and that CYK-4^{R459A}•RhoA•GTP complex does not effectively fulfill this role.

To obtain independent insight into how the CYK-4 GAP domain promotes cytokinesis, we identified mutations that suppress *cyk-4(or749)* to viability (*or749* has GAP domain mutation, causing cytokinetic failure). One isolate containing a substitution mutation in ECT-2 was proven to be causative. *ect-2(xs110)* mutant embryos exhibit hypercontractility during cytokinesis in a CYK-4-dependent manner. *ect-2(xs110)* and *cyk-4(or749)* mutually suppress. These results demonstrate that the primary defect in *cyk-4(or749)* is a failure to activate RhoA.

We show that the GAP domain of CYK-4 promotes ECT-2-dependent RhoA activation, and suggest that this activity is enhanced by RhoA binding to the GAP domain of CYK-4.

P210

Board Number: B322

MgcRacGAP's GAP activity spatially restricts active RhoA and Rac1 during cytokinesis and maintains proper adherens junction structure in epithelial cells.

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Cytokinesis is the last step of cell division where one cell is physically separated into two. In animal cells, localized activation of the small GTPase RhoA (RhoA-GTP) in a focused equatorial zone is essential for cytokinesis. Activation of RhoA is dependent on the guanine nucleotide exchange factor Ect2, which is spatially confined at the equatorial membrane by Centralspindlin, a complex of the kinesin MKLP1 and the GTPase activating protein (GAP) MgcRacGAP (Mgc). Both the identity of the GTPase targeted by Mgc's GAP activity and the question of whether phosphorylation of Mgc at serine 386 affects the specificity of Mgc's GAP activity are controversial. Here, using *Xenopus laevis* embryos as a model system, we examine for the first time Mgc's role in the intact vertebrate epithelium. We show that Mgc's GAP activity regulates the proper spatially restricted accumulation of both RhoA-GTP and Rac1-GTP during cytokinesis in epithelial cells. Phosphorylation at serine 386 within Mgc's GAP domain is not required for proper regulation of GAP activity toward RhoA or successful cytokinesis in the intact epithelium. However, perturbing Mgc's GAP activity leads to cytokinesis failure, abnormally increased accumulation of RhoA-GTP at the cleavage furrow, and increased local accumulation of both RhoA-GTP and Rac1-GTP at cell-cell junctions. Furthermore, Mgc regulates adherens junction but not tight junction structure, and Mgc's ability to regulate adherens junctions is dependent on its GAP activity. Cells expressing a Mgc GAP-dead mutant had defects in adherens junctions, which could be rescued by expressing dominant negative RhoA, suggesting that the defects are due to misregulated active RhoA. These results indicate that Mgc's GAP activity is necessary to down-regulate the active populations of RhoA at the division site and both RhoA and Rac1 at cell-cell junctions in epithelial cells, and failure to do so results in defects in both cytokinesis and cell-cell junctions.

P211**Board Number: B323****The GAP activity of *C. elegans* CYK-4 is required for incomplete cytokinesis in the syncytial germline but not for contractile ring constriction during embryonic cytokinesis.**K. Lee¹, R. Green¹, A. Desai¹, K. Oegema¹;¹Ludwig Institute for Cancer Research, La Jolla, CA

Contractility-based remodeling of the actomyosin cell cortex partitions nuclei into distinct cells during cytokinesis. An incomplete cytokinesis-like process also partitions nuclei into membrane-bound compartments that remain cytoplasmically connected in syncytial tissues such as the *C. elegans* germline and *Drosophila* embryo. Cortical remodeling during cytokinesis is directed by regulators concentrated on the anaphase spindle, including the Rho family GTPase-activating protein (GAP) CYK-4, a subunit of the centralspindlin complex, that also includes kinesin-6. CYK-4 is also required for nuclear partitioning in the germline and thus for organismal fertility. While the importance of CYK-4 is undisputed, the activities responsible for its specific functions are controversial. CYK-4 has two functional domains: (1) a GAP domain, predicted to target a Rho family GTPase, and (2) a C1 domain, which confers association with the plasma membrane. We have tested the importance of CYK-4 GAP activity as well as of the C1 domain in *C. elegans* by deleting or mutating each domain and analyzing embryonic cytokinesis and germline nuclear partitioning. As complete inhibition of CYK-4 prevents the generation of embryos, we engineered strains with an additional wild-type CYK-4 copy that is tagged with a degron that results in its elimination at the meiosis-to-mitosis transition to enable selective analysis of the consequences of engineered mutations on embryonic cytokinesis. All mutants in the GAP and C1 domains supported central spindle assembly. Deletions of either domain, or point mutations disrupting the C1 domain, disrupted contractile ring assembly and cytokinesis. However, mutation of GAP activity (by either a single point mutation in the catalytic residue R459A or a triple point mutation targeting the catalytic Arginine plus two other conserved residues important for GTPase binding: R459A/K495A/R499E: AAE) did not disrupt contractile ring assembly and constriction in the early embryo, although it did appear to compromise cytokinesis completion as abscission failed in ~16% of R459A and ~45% of AAE embryos. In contrast to embryonic cytokinesis, all mutants in the GAP and C1 domains, including the R459A and AAE GAP activity mutants, disrupted the incomplete cytokinesis-like process that partitions nuclei in the germline and severely reduced fertility. Thus, the GAP and C1 **domains** are essential for all CYK-4 functions, but GAP **activity** is required to promote abscission during embryonic divisions and for incomplete cytokinesis in the germline but not for contractile ring assembly and constriction.

P212**Board Number: B324****Role of GTP in Septin Assembly and Dynamics in *Ashbya gossypii*.**A. Khan¹, A. Gladfelter¹;¹Dartmouth College, West Lebanon, NH

Septins are GTP binding proteins that can come together to form rod-shaped hetero-oligomers. These rod complexes can give rise to filaments that organize into higher order structures. Septins have been shown to bind and hydrolyze GTP, however the relationship between septin assembly and GTP binding/hydrolysis is unclear. Here we use the filamentous fungus *Ashbya gossypii* to study the role of GTP in septin assembly and dynamics. In *Ashbya*, there are six mitotic septins encoded by CDC10, CDC3, CDC12, CDC11A, CDC11B and SHS1. While the position of most septins in the rod complex is fixed, Cdc11 and Shs1 can compete for the terminal position where they bind and interact with Cdc12. Using GTP binding mutants, we show that nucleotide binding can change Cdc12 binding partners in the septin rod complex, possibly by changing the oligomerization interfaces. This preference for Shs1 over Cdc11 changes the rod composition, which translates to behavioral changes of higher order structures *in vivo*.

P213**Board Number: B325****Protease-dead separase is dominant negative in the *C. elegans* embryo.**X. Bai¹, D. Mitchell¹, L. Uehlein¹, J.N. Bembenek¹;¹Univ Tennessee-Knoxville, Knoxville, TN

Separase is a protease that promotes chromosome segregation at anaphase by cleaving cohesin. Several non-proteolytic functions of separase have been identified in other organisms. We found that separase is required for vesicle exocytosis during anaphase, and hypothesize that its protease activity is required. To address this, we expressed protease-dead separase in *C. elegans* embryos. We find that expression of protease-dead separase is dominant-negative in embryos, not previously reported in other systems. The *C. elegans* embryo is an ideal system to study developmental processes in a genetically tractable system. However, a major limitation is the lack of an inducible gene expression system for the embryo. We have developed two methods that allow for the propagation of lines carrying dominant-negative transgenes and have applied them to characterize expression of protease-dead separase in embryos. Using these methods, we show that protease-dead separase causes embryo lethality, and that protease-dead separase cannot rescue separase mutants. Furthermore, expression of protease dead separase interferes with chromosome segregation and vesicle trafficking during cytokinesis. In addition, ectopic accumulation of protease dead separase is observed at several putative sites of action within the cell during anaphase, including the furrow and midbody. These data suggest that protease-dead separase interferes with endogenous separase function, possibly by binding substrates and protecting them from cleavage. Identification of relevant substrates will be an important goal to understand the mechanism of separase dependent exocytosis.

P214**Board Number: B326****Reconstituted Cytokinesis Signaling.**A.C. Groen¹, P.A. Nguyen¹, M. Loose², K. Ishihara¹, M. Wühr¹, T.J. Mitchison¹, C.M. Field¹;¹Department of Systems Biology, Harvard Medical School, Boston, MA, ²Harvard Med Sch, Boston, MA

Cell division is the process whereby one cell divides into two daughter cells and is essential for many aspects of biology, including growth, immune response, and cancer (when unregulated). Successful symmetric cell division relies on precise positioning of the cleavage furrow at the midplane. During early zygotic cell divisions, astral microtubules grow from the spindle poles out to the cortex after anaphase to anchor the spindle apparatus during chromosome segregation. We developed a novel cell-free system from frog egg extracts that reconstitutes growth and interaction of asters nucleated from artificial centrosomes. Similar to embryos, these zones recruit critical cytokinesis protein complexes, such as Kif4-PRC1, Centralspindlin, and the chromosomal passenger complex (CPC). Most importantly, we observed the recruitment of cleavage furrow markers (anillin, actin, and GTP-bound RhoA) at interaction zones assembled over supported lipid bilayers (which mimic the plasma membrane). This is the first time a spindle midzone-like structure was reconstituted that can signal to the cortex to initiate the cleavage furrow. We are currently exploring the role of microtubule dynamics on recruitment of cleavage furrow signals at the zone.

P215**Board Number: B327****Differential survivin expression at the single-cell level as revealed by flow cytometry and microscopy.**A. Miletic¹, W. Kang¹, B. Scott¹, X. Li¹, L. Ma¹, S.G. Becker-Catania¹, S. Lee¹, N. Schrantz¹, P. Just¹;¹Research and Development, eBioscience, An Affymetrix Company, San Diego, CA

Survivin (also known as Birc5) is a member of the inhibitor of apoptosis (IAP) family that also includes XIAP, c-IAP1, c-IAP2, and NAIP. Like other members of the IAP family, one major function of survivin is inhibition of caspase activation, which leads to the negative regulation of apoptosis. Nevertheless, survivin also plays a critical role in mitosis and cytokinesis, during which it is expressed only during the G2/M-phase and can be found localized to the mitotic spindle and cleavage furrow/midplate. Due to its role in cell cycle progression, survivin has been found to be highly expressed in tumors and fetal tissue, but is absent in terminally-differentiated and non-proliferating cells. Moreover, deletion of survivin in developing mouse thymocytes, as well as in mature T and B cells, demonstrated that survivin is critical for cell division in lymphoid cells but not for survival. To date, very few tools are available to detect and visualize survivin expression and localization *ex vivo*. Here, we describe a novel mouse monoclonal antibody that specifically recognizes human survivin and demonstrate its utility in flow cytometry and immunocytochemistry to examine survivin expression in actively proliferating T cells. With this antibody,

researchers will be able to gain a better understanding of the role survivin plays in the balance between cell proliferation and apoptosis during cancer and development.

G1, G1-S, and S Phase Regulation

P216

Board Number: B328

Effect of pituitary tumor transforming gene (PTTG1) on the self-renewal activity of placenta-derived mesenchymal stem cells.

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In addition to differentiation potential, self-renewal capability is an important characteristic of stem cells. The limited self-renewal activity of mesenchymal stem cells is the biggest obstacle to the application of stem cell therapy in regenerative medicine. The human TERT gene enhances the self-renewal of MSCs, but the mechanism of self-renewal and the interactions among TERT gene-related molecules remain unknown. The objectives of this study were to generate immortalized MSCs derived from mesenchymal stem cells isolated from placenta (Naïve) by human TERT gene transfection using the AMAXA gene delivery system, compare their characteristics, and investigate whether increased TERT expression affects the pituitary tumor transforming gene (PTTG1) (also known as securin), which is involved in chromosome segregation during mitosis. TERT-immortalized cells (TERT+) with prolonged life span displayed high PTTG1 expression. TERT+ cells also retained the stemness capacity and multipotency of naïve cells and displayed high PTTG1 expression. However, down-regulation of PTTG1 by treatment with siRNA induced cell senescence and decreased telomerase activity. In addition, TERT bound to PTTG1 forms complex with chaperones such as Ku70 and heat shock protein 90 (HSP 90). Taken together, placental MSCs immortalized by TERT gene transfection display differentiation potential and enhance the self-renewal of MSCs through a balanced interaction of PTTG1 and chaperones. The results suggest that the interaction between TERT and PTTG1 by association of Ku70 could be important for the enhancement of the limited self-renewal activity of MSCs as well as for understanding the regulatory mechanisms of self-renewal.

P217**Board Number: B329****Cell Cycle Dependent Transcription is Regulated by Nuclear Positioning in a Syncytium with Asynchronous Nuclear Division.**S.E. Roberts¹, A.S. Gladfelter¹;¹Biological Sciences, Dartmouth College, Hanover, NH

In multinucleate cells of the fungus *Ashbya gossypii*, nuclei divide independently despite sharing a common cytoplasm. Internuclear distance is tightly regulated, with neighboring nuclei ~5 μm apart. We demonstrated that heterogeneous localization of cyclin transcripts by their association with protein aggregates promotes asynchronous division. To determine whether variability in the production of transcripts also promotes transcript spatial heterogeneity, nuclei producing cyclin transcripts were identified using single molecule RNA FISH. Cyclin transcription correlated with the cell cycle phase of the nucleus indicating that even in a shared cytoplasm nuclei have “knowledge” of their cell cycle phase. It is possible that variable transcription of cyclins contributes to variable local cytosolic concentrations of cyclin protein. If so, the volume of cytosol between neighboring nuclei may be crucial to nuclear cycle autonomy. In fact, previous data showed when nuclei cannot control their spacing they undergo synchronous division. To further investigate the relationship between nuclear spacing and division autonomy we utilized a mutant strain lacking the dynactin complex in which large nuclear clusters form with 10-30 nuclei

P218**Board Number: B330****IRBIT is a novel regulator of Ribonucleotide reductase in high eucaryotes.**M. Dasso¹, A. Arnaoutov¹;¹National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD

Ribonucleotide reductase (RNR) is an enzyme that supplies the balanced pools of deoxynucleotide triphosphates (dNTPs) necessary for DNA replication and maintenance of genomic integrity. RNR is subject to allosteric regulatory mechanisms in all eukaryotes, as well as to control by small protein inhibitors Sml1p and Spd1p in budding and fission yeast, respectively. We have found that the metazoan protein IRBIT controls RNR activity via a novel allosteric mechanism: IRBIT forms of a complex with RNR in dATP-dependent manner, stabilizing dATP in the activity site of RNR, and thus inhibiting the enzyme. Formation of the RNR-IRBIT complex is regulated through phosphorylation of IRBIT, and ablation of IRBIT expression in HeLa cells causes imbalanced dNTP pools and altered cell cycle progression. We have begun to develop model systems for examination of IRBIT function in intact organisms, and have particularly focused on analysis of the *Drosophila melanogaster* midgut, a well-characterized system for tissue generation and homeostasis. Our preliminary findings suggest that these processes are misregulated in the absence of IRBIT.

P219**Board Number: B331****Ran GTPase activates DNA damage response and cell cycle resumption, driving the evasion of cell senescence in normal and cancer cells.**P. Cekan¹, S. Ryu^{1,2}, K. Hasegawa^{1,3}, J. Lee¹, P. Kalab¹;¹NCI/NIH, Bethesda, MD, ²Well-Aging Research Center, Samsung Advanced Institute of Technology, Osan, Korea, ³Biochemistry-Biophysics, Amherst College, Amherst, MA

Several lines of evidence indicated that the small GTPase Ran could have a role in activating cell cycle resumption in the interphase. First, Ran is required for the nuclear-cytoplasmic transport of cell cycle regulators, including nuclear export of cyclin mRNAs and nuclear import of DNA replication factors. Second, the activity of Ran-regulated nuclear transport was found to be reduced in non-dividing senescent cells. Finally, previously we showed that rapidly dividing tissue culture cells expressed increased levels of RCC1, the factor required for the formation of the GTP-loaded active form of Ran. Here we show that stable cell cycle exit in normal senescent cells led to the decline of RCC1 expression and reduced RanGTP levels. In contrast, RCC1 expression was required for cell cycle resumption in colorectal carcinoma cells recovering from DNA damage-induced senescence-like state. Remarkably, RCC1 overexpression was sufficient to override the DNA damage-induced cell cycle arrest in normal epithelial cells, leading to the rise of aneuploid proliferative cells. The effects of RCC1 on cell senescence evasion in normal and cancer cells required the role of RanGTP and Exportin 1 in nuclear export and involved the activated expression of many factors promoting DNA damage repair, DNA replication and cell cycle. These results indicate that, through its interphase nuclear functions, RanGTP promotes DNA damage repair and cell cycle initiation, biasing the cellular response to genotoxic insults towards continuous proliferation in the presence of severe genomic alterations.

P220**Board Number: B332****Elm1 regulates Hsl1/Hsl7 interaction in the morphogenesis checkpoint.**H. Kang¹;¹Pharmacology, Duke University, Durham, NC

The yeast morphogenesis checkpoint prevents nuclear division prior to bud emergence through Swe1-mediated inhibition of Cdc28. After bud formation, Swe1 is targeted for degradation via sequential phosphorylation events that require localization of the kinases Elm1 and Hsl1, as well as the Swe1-binding protein Hsl7, to septin ring at the mother-bud neck. Elm1 activates Hsl1, which recruits Hsl7 to septin ring only in budded cells. How do these proteins “know” when budding has occurred? By tethering the small Hsl7-binding domain of Hsl1 to a septin, we found that this domain is sufficient to confer Hsl7 recruitment. Although the Hsl1 fragment was present before budding, Hsl7 was only recruited after budding. The Elm1 kinase was also localized to the septins only after budding. Using a Bni4-Elm1 fusion that targets the Elm1 kinase to the septin ring prior to budding, we found that

premature Elm1 targeting led to premature Hsl7 recruitment by the Hsl1 fragment. Over expression of Elm1 leads to its early localization before budding. Similarly, early localized Elm1 can also contribute to Hsl7 recruitment by Hsl1 fragment in unbudded cells. These findings suggest that Elm1 localization is the process that coordinates with bud emergence, and that it regulates Hsl1/Hsl7 interaction to promote Hsl7 recruitment and Swe1 degradation only after budding has begun.

P221

Board Number: B333

Yeast metabolic cycle: Entry into oxidative phase is tightly coupled to the cell cycle in diverse strains and growth conditions.

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The budding yeast *Saccharomyces cerevisiae* is capable of synchronous metabolic oscillations with a period between 45 minutes and several hours when grown in chemostat conditions. This oscillation consists of alternating periods of buildup and oxidation of the storage carbohydrates trehalose and glycogen, and changes in expression of a large fraction of the transcriptome. The cell division cycle (CDC) couples strongly to this oscillation, with a single pulse of cells entering S phase once per yeast metabolic cycle (YMC). Early work showed that in some strains DNA replication and oxidative phase never overlapped, presumably to prevent damage to DNA during replication. However, more recent work in other strains has shown that DNA replication can occur both during and outside of oxidative phase.

To better understand strain-dependent and strain-independent phenotypes, we examined YMC-CDC coupling in different laboratory strains and wild-isolates across varying chemostat conditions. We show that all strains exhibit a linear relationship between YMC and CDC period, that oxidative phase can overlap DNA replication in many strains, and that the interval between entry into oxidative phase and DNA replication is invariant for a given strain across growth conditions. These data suggest that cell cycle Start is tightly coupled to events surrounding the entry into the high-metabolic-rate oxidative phase. This excludes mechanisms and rationales for cell cycle coupling involving protection of DNA from metabolic damage, and instead suggests a role for coupling in ensuring DNA replication occurs when sufficient cellular energy is available. Moreover, when combined with recent work revealing direct metabolic effects on cyclin gene expression it suggests a positive feedback loop between carbon metabolism and cell cycle Start which synchronizes compatible cellular and metabolic events to ensure they occur together.

P222**Board Number: B334****The anaphase-promoting complex works together with the SCF complex for proteolysis of the S-phase cyclin Clb6 during the transition from G1 to S phase.**S. Wu¹, V.J. Kuan², Y. Tseng³, S.C. Schuyler², Y... Juang⁴;¹Microbiology and Immunology, Tzu-Chi University, Hualien, Taiwan, ²Biomedical Sciences, Chang Gung University, Taoyuan, Taiwan, ³Institute of Medical Sciences, Tzu-Chi University, Hualien, Taiwan,⁴Mackay Medical College, New Taipei City, Taiwan

In *Saccharomyces cerevisiae*, the S-phase cyclin Clb6 is expressed shortly at the G1/S border. It has been shown that the SCF^{Cdc4} ubiquitin ligase controls Clb6 proteolysis requiring cyclin-dependent kinases and that Clb6-3A, bearing non-phosphorylatable mutations of S6A, T39A, and S147A, is hyperstabilized but still unstable in mitosis. In this study, we found that the APC^{Cdh1} form of anaphase-promoting complex, an E3 ubiquitin ligase, was required for Clb6 proteolysis in both early and late G1. *In vitro* ubiquitination assay further confirms that Clb6 is a substrate of the APC^{Cdh1}. Clb6 has a KEN box and a destruction box in its N-terminus. Mutations in the KEN box (mkb) and/or the destruction box (mdb) could enhance Clb6 stability in G1. Clb6^{mkd} bearing mutations of mkb and mdb could make cells bypass the late G1 arrest caused by the *cdc4-1* temperature-sensitive mutation depending upon CDK phosphorylation at residues S6, T39 and S147. As compared to Clb6, overexpression of Clb6-ST, bearing mutations of S6A, T39A, S147A, mkb, and mdb, had a greater effect on promoting expression of Clb2 and S-phase entry, and caused a greater G2 delay and a greater defect in cytokinesis. Furthermore, Swe1 was required for occurrence of bud emergence when Clb6-ST was overexpressed. Overall, our observations suggest that both APC^{Cdh1}- and SCF^{Cdc4}-dependent proteolysis of Clb6 at the G1/S border is crucial for proper expression of Clb2, G1/S transition, G2/M transition, and cytokinesis.

P223**Board Number: B335****Cyclin oscillations in living cells: Endogenous reporters for a quantitative view on cyclin expression and proteolysis.**M. Lachnit¹, I. Gak¹, J. Mansfeld¹;¹Cell Cycle, Biotechnology Center, Technische Universität Dresden, Dresden, Germany

Cell cycle progression is tightly controlled by oscillations of cyclins that activate distinct cyclin-dependent kinases (Cdk) at the right time. While the protein level of the Cdk generally remains constant, the levels of cyclins are precisely controlled by 'tug of war' between cyclin expression and ubiquitin-mediated proteolysis. Decades of research have revealed the key players and pathways regulating cyclin stability. Little is known, however, about the *in vivo* dynamics of cyclin oscillations and their response to cell cycle challenges due to the lack of reporters that faithfully reflect the regulation of endogenous cyclins. Using non-transformed retinal pigment epithelial cells as a model we have here employed

homologous recombination techniques to insert the gene of the fluorescent protein mVenus site-specific and in frame into the loci of cyclins A2, B1 and D1. Notably, the fluorescent fusion proteins behave comparably to the untagged cyclin indicating that measuring mVenus fluorescence is a valid readout to visualize cyclin oscillations in living cells. To relate the fluorescence data to the exact cell cycle stage we have further tagged endogenous the proliferating cell nuclear antigen (PCNA) and histone H3.1 with mRuby and mTurquoise2, respectively. Therefore, we can automatically segment and classify single cells into the exact cell cycle stage. Furthermore, the same fluorescent tag on different cyclins allows us to precisely determine their absolute and relative expression and proteolysis kinetics by quantitative live cell microscopy. As expected and validating this approach, cyclins A2 and B1 are rapidly degraded in mitosis and accumulate in S- and G2-phase, respectively. Cyclin D1 in contrast to the prevalent view oscillates in G1-phase, strongly decreases before S-phase, and then re-accumulates in G2-phase. Allowing nuclear segmentation (histone), cell cycle stage classification (PCNA) and cyclin expression (mVenus)- all dependent on endogenously-tagged proteins - the cyclin reporters will be valuable tools to get a more quantitative view on cyclin oscillations and to assay the behaviour of single cells when the cell cycle is challenged.

P224

Board Number: B336

Phase separated RNA-protein droplets position cyclin transcripts to organize the cytosol of multinucleate cells.

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Syncytial cells are found throughout the biosphere from filamentous fungi to early animal development, muscle, placenta and tumor cells. Multinucleate cells face unique organizational problems and in many cases must functionally compartmentalize their cytosol for diverse processes including nuclear division and symmetry breaking. We found that in the filamentous fungus *Ashbya*, in which nuclei cycle asynchronously despite sharing a cytosol, that cyclin transcripts are clustered and kept near nuclei by binding to the RNA-binding protein, Whi3. Whi3 has a substantial polyQ tract and this aggregation-prone region is essential for clustering transcripts and asynchronous nuclear division. We hypothesized that Whi3-cyclin complexes form functional, dynamic aggregates that phase separate from bulk cytosol, potentially to control the location of cyclin protein production. We have reconstituted the cyclin transcript-polyQ protein complex and found that at physiological concentrations Whi3 and cyclin mRNAs phase separate and form liquid droplets both in buffer and in concentrated cell extracts. Biophysical measurements of the droplet properties indicate that they are dynamic, fluid and highly dependent on having mRNA and full length Whi3 protein. Similarly, in cells the ability of Whi3 protein to bind mRNA is essential for its heterogeneous localization into dynamic puncta and tubules, as well as nuclear asynchrony. These results demonstrate a function for RNA-protein granules in regulating variable and asynchronous nuclear progression and show mRNA is a critical component for the assembly process. We

propose that phase-separated droplets may be widely employed to pattern cytosol for diverse processes beyond the regulation of mRNA turnover and are likely critical for large, syncytial cell organization.

P225

Board Number: B337

The circadian factor Period 2 modulates p53 stability and transcriptional activity in unstressed cells.

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Circadian rhythms are mechanisms that measure time on a scale of about 24 h and that adjusts our bodies to external environmental signals. Core circadian clock genes are defined as genes whose protein products are necessary components for the generation and regulation of circadian rhythms. Disruption of circadian rhythm has recently emerged as a new potential risk factor in the development of cancer, pointing to the core gene *period 2 (per2)* as a tumor suppressor. However, it remains unclear how the circadian network regulates tumor suppression, nor which, if any, of its components is either the ultimate effector that influences the fate of the cell. The relevance of hPer2 to human disease is underlined by alterations in its function that impacts numerous biochemical and physiological processes and, when absent, result in the development of various cancers. We found hPer2 binds the C-terminus half of human p53 (hp53) and forms a stable trimeric complex together with hp53's negative regulator Mdm2. We determined that hPer2 binding to hp53 prevents Mdm2 from ubiquitinating and targeting hp53 by the proteasome. Accordingly, downregulation of hPer2 expression directly impacts hp53 levels whereas its overexpression influences both hp53 protein stability and transcription. Furthermore, we spatially defined the distribution of the trimeric complex within the nucleus. Lastly, we established that hPer2 binding influences hp53-mediated gene transcription (e.g., *CDKN1a*, *SFN*, *GADD45α*) in response to DNA-damage by reconstituting the p53-mediated signaling pathway in a p53^{-/-} cellular model. Overall, our findings place hPer2 directly at the heart of the hp53-mediated response by ensuring that basal levels of hp53 are available to rapidly respond to a stress conditions.

P226

Board Number: B338

Functions of PP1-NIPP1 in cell cycle progression.

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Protein Ser/Thr phosphatase 1 (PP1) forms heterodimers with dozens of structurally unrelated polypeptides that regulate substrate selection and enzymatic activity. One of the major nuclear interactors of PP1 is NIPP1, which has a ForkHead-Associated (FHA) domain that recruits a subset of

Cdk-phosphorylated proteins for dephosphorylation by associated PP1. To delineate the role of PP1-NIPP1 in cell-cycle progression we have generated cell lines (Flp-In T-REx HeLa) that inducibly express NIPP1 variants or fusions. The expression of NIPP1 caused a block in early-mid-mitosis associated with lagging chromosomes, spindle defects and a permanently activated spindle-assembly checkpoint, often culminating in mitotic cell death. This heterogeneous phenotype was also observed after expression of a FHA-binding mutant of NIPP1 but was completely absent after expression of a PP1-binding mutant. Additional data suggested that the mitotic defects induced by the overexpression of NIPP1 stem from the competitive disruption and inhibition of PP1 holoenzymes that are essential for mitotic progression. Hence, NIPP1 expressing cell lines can be used to further delineate the mitotic functions of PP1. A completely different phenotype was observed after the expression of a PP1-NIPP1 fusion. This fusion caused replication stress, associated with the accumulation of γ H2AX foci and impaired DNA repair via homologous recombination. This effect was dependent on both a functional FHA domain and PP1 activity, and could be explained by the dephosphorylation and destabilization of two FHA ligands of NIPP1 that function in homologous recombination, namely protein kinase Melk and the tumor suppressor protein CtIP. Our data show that PP1-NIPP1 contributes to the reversal of Cdk-mediated cell-cycle progression.

P227

Board Number: B339

The deubiquitinase USP37 promotes the efficient S-phase progression and the cellular response to replication stress.

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Ubiquitin-mediated proteolysis is a key regulatory process in cell cycle progression. Recently we identified the deubiquitinase USP37 as regulator of S-phase entry. Here we report that depletion of USP37 leads to diminished cellular proliferation and loss of viability. USP37-depleted cells exhibit altered replication kinetics and increased levels of DNA damage markers γ H2AX and 53BP1. Moreover, USP37-depleted cells display significantly increased sensitivity to replication stress. Consistent with this increased sensitivity, activation of the checkpoint kinase Chk1 is attenuated in USP37-depleted cells. We have determined that decreased stability of the replication checkpoint mediator Claspin underlie the defects in Chk1 activation. USP37 interacts with and deubiquitinates Claspin. Our data suggest a model whereby temporally regulated interplay of USP37, APC^{Cdh1}, and the replication checkpoint machinery determines the timing of replication checkpoint activity that sharpens the G1/S transition and promotes the efficient completion of replication. These data provide an improved understanding of the replication checkpoint, control of APC^{Cdh1}, and maintenance of genome stability.

P228**Board Number: B340****Survival of proliferative, radio-resistant polyploid cells in *Drosophila* requires FANCD2.**H. Bretscher¹, D. Fox¹;¹Pharmacology and Cancer Biology, Duke University School of Medicine, Durham, NC

Maintaining a stable genome prevents damaged DNA, altered cellular function, and ultimately diseases such as cancer. Genome instability can result from a wide variety of cellular stresses including DNA damage. In order to prevent propagation of genome instability, diploid mitotic cells employ a DNA damage checkpoint to ensure that they do not divide with damaged DNA. Entering mitosis with damaged DNA can result in mutations and chromosome number imbalance (aneuploidy). While the response to DNA damage is well characterized in diploid mitotic cells, it has been recently observed that passage through the endocycle, a variant cell cycle in which mitosis is truncated, can alter the response to DNA damage. As a result of this alteration, endocycling murine trophoblasts and several *Drosophila* larval tissues are highly resistant to DNA damage. Unlike diploid cells, these non-proliferative cells continue to endocycle despite high levels of DNA damage.

While, most endocycled cells are terminally differentiated and do not re-enter the mitotic cell cycle, we previously established a model to address mechanisms by which the endocycle promotes genome instability during mitosis. In the *Drosophila* rectum, we found endocycled papillar cells can re-enter mitosis as polyploid cells. We show that, like other endocycled cells, papillar cells acquire radio-resistance during the endocycle. Rather than undergoing full repair of DNA breaks, papillar cells re-enter mitosis with broken chromosomes. Despite frequent DNA breaks, lagging DNA during anaphase and organism-lethal amounts of DNA damage, papillar cells develop normally and undergo surprisingly little cell death. To survive, we find that papillar cells enlist a non-canonical DNA damage mechanism to ensure that anaphase is extended to allow for incorporation of broken DNA into daughter nuclei. This survival depends on ATM and ATR, but not chk1 and chk2 kinase activity or the transcription factor p53. In addition to ATM and ATR, we find that the DNA repair protein FANCD2 is also required for cytokinesis delay and survival of damaged polyploid papillar cells. Cells lacking FANCD2 fail to incorporate broken DNA into daughter cell nuclei prior to the onset of cytokinesis, and subsequently die by mitotic catastrophe. We speculate that the mechanism used by polyploid papillar cells to survive mitosis with high levels of DNA damage may contribute to radio-resistance in cancer cells that have undergone an endocycle.

P229**Board Number: B341****Store-operated calcium entry (SOCE) controls G1/S cell cycle transition.**Y. Chen¹, M. Shen^{1,2};¹Department of Pharmacology, National Cheng Kung University Hospital, Tainan, Taiwan, ²Department of Obstetrics and Gynecology, National Cheng Kung University Hospital, Tainan, Taiwan

Background: The well-defined store-operated Ca²⁺ entry (SOCE) pathway is a ubiquitous Ca²⁺ influx pathway activated in response to depletion of intracellular Ca²⁺ stores. The major components of SOCE are stromal-interacting molecule (STIM1, STIM2) and Orai (Orai1, Orai2, Orai3). STIM1 serves a dual role as the endoplasmic reticulum Ca²⁺ sensor, and activator of SOCE. The clinical relevance of STIM1 has been highlighted in several cancers but the molecular mechanism by which SOCE involved in G1/S transition of cell cycle progression remains unclear. However, this study explores the regulatory mechanisms by which STIM1-dependent Ca²⁺ signaling controls cell cycle progression. **Results:** Here, we showed that SOCE increased in the S phase of cell cycle in different cell lines. Ca²⁺ microdomain is necessary for G1/S transition. We discovered that SOCE activity is necessary for G1/S transition but not important for S to G2/M transition. SOCE inhibitor, SKF96365 significantly inhibited G1/S transition to a similar extent to that of components of SOCE (STIM1, STIM2, Orai1) silencing. Total internal reflection fluorescence (TIRF) microscopy of living cells indicated that the increase of STIM1 trafficking to the plasma membrane in S phase but not in G0/G1 phase, suggesting STIM1 is important for the regulation of the G1/S transition. Inhibition of SOCE induced cyclin E autophagy as well as blockage of G1/S transition induced cell apoptosis. Taken together, our results revealed that the novel role of SOCE activity in controlling cell cycle progression.

P230**Board Number: B342****DRG2 binds to Wee1 for regulating cell cycle progression during G2/M phase.**S. Jang¹, N. Park², S. Kim³, J. Park⁴, I. Han⁴;¹Biomedical Research Center, University of Ulsan, Ulsan, Korea, ²Department of Medical Science, University of Ulsan, Ulsan, Korea, ³Department of biological sciences, Univeristy of Ulsan, Ulsan, Korea, ⁴Department of Biological Sciences, University of Ulsan, Ulsan, Korea

Developmentally regulated GTP-binding protein 2 (DRG2) plays important role in cell growth. Here we explored that the linkage between DRG2 and G2/M phase checkpoint function in the cell cycle progression. We observed that HeLa cell lacking DRG2 has the slower wound-healing rate than that of control cells. Flow cytometry assay and ³H incorporation assay indicated that G2/M phase arrest leads to decreased proliferatio of this cell. Depletion of DRG2 elicited the changed expression of major cell cycle determinant factors, such as cyclin A2, B1, p21^{Waf1/Cip.1}. In addition, DRG2 interacts with Wee1 kinase, a mitotic gatekeeper. These findings elucidate a noble role for DRG2 as regulator of Wee1, and therefore serve to progress G2/M phase in cell cycle.

P231**Board Number: B343****Phosphorylation of Xenopus p31comet potentiates mitotic checkpoint exit.**M. MO¹, M. Dasso², A. Arnaoutov²;¹National Institute of Health, Bethesda, MD, ²National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD

The spindle assembly checkpoint (SAC) ensures faithful segregation of mitotic sister chromatids by monitoring interactions between kinetochores (KTs) and microtubules (MTs). Defective MT-KT interactions trigger SAC activation, causing the formation of a potent inhibitory complex, the mitotic checkpoint complex (MCC), which in turn blocks the metaphase-anaphase transition. Once all KT have formed correct MT attachments, the SAC is silenced. The MCC is then disassembled through a series of poorly understood processes, allowing anaphase to ensue. It has previously been shown that the p31comet protein helps to inactivate the MCC by binding directly to Mad2, an essential MCC component. However, p31comet's regulation and mechanism of action are unclear. Xenopus egg extracts (XEEs) are an excellent in vitro system that recapitulates many aspects of cell cycle control, including the SAC. We confirmed that Xenopus p31comet was important for SAC silencing in XEEs, and observed that it was phosphorylated during the course of mitosis. Unexpectedly, our data suggest that this modification was mediated by IKK- β (Inhibitor of nuclear factor κ -B kinase-beta), but not by a panel of other well-known mitotic kinases. Depletion or chemical inhibition of IKK- β compromised SAC silencing and delayed mitotic exit, suggesting that this modification was important for p31comet regulation. We identified p31comet phosphorylation sites, and found that a p31comet phosphomimetic mutant (p31comet-EEE) shows enhanced binding of Mad2, an event critical for MCC disruption. Moreover, p31comet-EEE exhibited increased ability to disrupt MCC in XEEs, and it accelerated mitotic exit. Notably, p31comet-EEE also disrupted the association of SAC components to KT, where MCC is formed, in a dosage dependent manner. By contrast, wild type p31comet or a phosphodeficient mutant (p31comet-AAA) had no effect on KT-associated MCC. Taken together, our data indicate that phosphorylation of p31comet by IKK- β potentiates its ability to silence the SAC and may contribute to SAC silencing at the KT.

P232**Board Number: B344****Structure of cohesin subcomplex pinpoints direct shugoshin-Wapl antagonism in centromeric cohesion.**G. Zheng¹, K. Hara^{1,2}, Q. Qu¹, H. Liu¹, Z. Ouyang¹, Z. Chen¹, D. Tomchick¹, H. Yu^{3,4};¹University of Texas Southwestern Medical Center, Dallas, TX, ²School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka, Japan, ³Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX, ⁴Howard Hughes Medical Institute, Dallas, TX

Sister-chromatid cohesion, mediated by ring-shaped cohesin complex, is a prerequisite for proper chromosome segregation and genomic stability. Errors in chromosome segregation cause chromosome aberrations and aneuloidy, which can promote tumorigenesis.

Human cohesin complex consists of four core subunits, Smc1, Smc3, Scc1, SA1/2. The loading and release of cohesin from chromosomes are tightly regulated during the cell cycle. During S phase, cohesin core subunit Smc3 is acetylated by the acetyltransferase Escp1/2, which enables the binding of the cohesin protector Sororin to cohesin through the adaptor protein Pds5. Sororin antagonizes the cohesin inhibitor Wapl to stabilize cohesin on replicated chromatin and establishes sister chromatid cohesion. In prophase, mitotic kinases phosphorylate cohesin and Sororin, triggering Wapl-dependent cohesin removal from chromosomes. A pool of cohesin at centromeres is protected by the Sgo1-PP2A complex, which binds to cohesin, dephosphorylates Sororin, and protects cohesin from Wapl at centromeres. After all sister kinetochores attach properly to the mitotic spindle and are under tension, Separase cleaves centromeric cohesin to initiate sister chromatid separation. The separated chromatids are evenly partitioned into the two daughter cells.

We reported the crystal structure of human cohesin subcomplex SA2-Scc1. SA2 has multiple helical repeats, which form a dragon-shaped structure. Scc1 makes extensive contacts with SA2, with one hot spot being particularly critical for binding. SA2-Scc1 complex is the interaction hub for cohesin regulators. Cohesin protector Sgo1 and inhibitor Wapl compete for binding to a conserved site on SA2-Scc1. Mutations of SA2 residues at this site that disrupt Wapl binding bypass the requirement for Sgo1 in cohesion protection. Thus, in addition to recruiting PP2A to dephosphorylate cohesin and sororin, Sgo1 physically shields cohesin from cohesin inhibitor Wapl. These two mechanisms collaborate to protect centromeric cohesion to the fullest extent, which makes centromeric cohesion strong enough to resist the spindle pulling force at sister kinetochores and enables the generation of kinetochore tension necessary for spindle checkpoint inactivation and accurate chromosome segregation.

Sgo1 is also known to protect meiotic cohesin at centromeres from separase-mediated cleavage during meiosis I. In preliminary results, I have shown that SA1/2, as the interaction hub, is required for separase cleavage of Scc1 at anaphase onset. It is possible that separase is recruited by SA1/2 to cleave centromeric cohesin. Overexpression of Sgo1 reduces the separase cleavage efficiency. This indicates that Sgo1 might directly compete with separase for binding SA1/2 and shield cohesin from separase. To

test this hypothesis, cohesin cleavage by separase will be reconstituted in vitro using purified proteins. Cohesin cleavage efficiency will be compared in the presence and absence of Sgo1 peptide.

P233

Board Number: B345

Actin-capping proteins play essential roles in spindle migration and polar body protrusion in maturing mouse oocytes.

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Actin polymerization is essential for various stages of mammalian oocyte maturation, including asymmetric spindle migration, cortical actin cap formation, polar body extrusion, and cytokinesis. The heterodimeric actin capping protein (CP) is an essential element of the actin cytoskeleton. It binds to the fast-growing (barbed) ends of actin filaments, thereby blocking their elongation, and plays essential roles in various actin-mediated cellular processes. However, the roles of CP in mammalian oocyte maturation are poorly understood. We investigated the roles of CP in mouse oocytes, and found that CP is essential for correct asymmetric spindle migration and polar body extrusion. Experiments in which CP was knocked down or ectopically overexpressed revealed that this protein is critical for maintenance of cytoplasmic actin mesh. Perturbations in the level of CP impaired spindle migration, cytoplasmic actin mesh and polar body extrusion during oocyte maturation. Expression of capping protein inhibitory region of CARMIL reduce polar body extrusion rate of oocyte and significantly reduce cytoplasmic actin mesh. Taken together, this study shows that CP is an essential component of the actin cytoskeleton machinery, presumably controlling cytoplasmic actin mesh during oocyte maturation.

P234

Board Number: B346

Ptychography: Label free imaging of the cell cycle and mitosis.

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The use of fluorescent probes has become commonplace for staining fixed biological specimens; however when used in living cells they can be toxic and ultimately perturb the natural function of the cell. With this in mind, there has been an emerging need for label free microscopy. Existing techniques such as Zernike phase contrast and DIC have limitations and most importantly are not quantitative. Here we report a novel stain-free, high-contrast and quantitative method for imaging live cells using a ptychographic microscope (Phasefocus VL21). The technique reconstructs an image from overlapping diffraction patterns using a ptychographical algorithm (Maiden et al., 2010), to give high contrast, quantitative information of the sample.

We have previously demonstrated the power of ptychography to quantitatively analyse cells during mitosis (Marrison et al., 2013). Here we demonstrate further measurement of the mitotic index (MI) of A549 cells in response to the cell cycle inhibitor, Nocodazole. An advantage to this imaging approach is the ability to acquire large fields of view in which data from 500+ cells can be analysed in a manner comparable to flow cytometry. Following the 6hr treatment with 10, 25 and 50ng/ml Nocodazole, the MI value also increased to 0.05, 0.23 and 0.36 respectively when compared to the control (MI= 0.03).

Furthermore we were able to use ptychography to analyse the cell cycle yielding phase characteristics related to cell thickness, cell volume and nuclear volume. Following mitosis the volume of the daughter cell increases linearly during the cell cycle and approximately doubles immediately prior to the next mitotic event. We also propose that nuclear volume remains constant through G1, but starts to increase during S phase. There is an increasing need for quantitative label-free imaging methodologies. Here we demonstrate that ptychography generates high contrast and quantitative images that not only enable cell cycle analysis using a label free non-invasive approach, but can now be used in studies where cell volume/mass are underpinning the biological function of the cell. In conjunction with in situ fluorescence imaging, the quantitative phase information can add additional and vital information that goes beyond fluorescence imaging itself.

In summary, the work highlighted here demonstrates one potential application of label-free phenotypic analysis of cells. The method is ideally suited for primary and stem cells where labels are undesirable or not possible. Furthermore the technique could also be used in many other assay types, such as anti-cancer therapeutics, cell motility and migration, cell morphology and neurodegeneration.

P235

Board Number: B347

Identifying candidate molecules through which γ -tubulin acts to inactivate the anaphase promoting complex/cyclosome at G1/S in *Aspergillus nidulans*.

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In addition to its well-established role in nucleating microtubules at microtubule organizing centers such as the centrosome and spindle-pole body, γ -tubulin has additional, essential, microtubule-independent functions that are incompletely understood. Experiments with the cold-sensitive γ -tubulin mutant *mipAD159* in the filamentous fungus *Aspergillus nidulans* revealed that γ -tubulin has an important role in inactivating the active anaphase promoting complex/cyclosome (APC/C) bound to the activator CdhA (the *A. nidulans* Cdh1 homolog) at the G₁/S transition (Edgerton-Morgan and Oakley, 2012, *J. Cell Biol.* **198**, 785-791). As a result, cyclin B is continuously destroyed in a subset of nuclei and these nuclei do not progress through the cell cycle (Nayak et al., 2010, *J. Cell Biol.* **190**, 317-330). The goal of this project is to elucidate the mechanism by which γ -tubulin inactivates APC/C^{CdhA}. Based on our data so far, we hypothesize that γ -tubulin acts through regulators of CdhA. In many organisms, initial phosphorylation and inactivation of Cdh1 occurs via cyclin A/Cdk2, which is then thought to trigger Cdh1 ubiquitination

by the Skp1-Cullin1-F-box (SCF) complex. We have identified a putative *A. nidulans* cyclin A gene, which we designate *cycA*, and a component of the SCF complex, designated *culA*, and found both to be essential for viability. If CycA is required for inactivation of APC/C^{CdhA}, the deletion of *cycA* (*cycAΔ*) is expected to result in lethality due to a continuously active APC/C^{CdhA}. It follows that deletion of *cdhA* might reverse the lethality of the *cycAΔ* and we found that this is the case. We have examined the phenotypes of *cycAΔ* and *culAΔ* using the heterokaryon rescue technique. *cycAΔ* inhibited nuclear division as expected, but, more interestingly, caused nuclei to disappear over time (as judged by fluorescently tagged histone H1) raising the possibility that *cycAΔ* may cause apoptosis. Additionally, we have found that a subset of nuclei fail to accumulate Cdk1 in *culAΔ*, similar to a phenotype observed in *mipAD159*, suggesting that APC/C^{CdhA} is constitutively active in these nuclei. Deletion of *culA* likely prevents degradation of CdhA, resulting in nuclei that accumulate constitutively active APC/C^{CdhA} and fail to progress through the cell cycle. Taken together, our data indicate that CdhA is regulated by CycA and the SCF complex in *A. nidulans* as in higher eukaryotes. They also indicate that CycA and CulA are valid candidates for proteins through which γ -tubulin acts to control APC/C^{CdhA} activity. Supported by the Irving S. Johnson Fund of the KU endowment.

P236

Board Number: B348

A tale of two phases: How RNA-protein interactions can spatially position transcripts involved in cell cycle control and morphogenesis.

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In the multinucleate cells of the fungus, *Ashbya gossypii*, individual nuclei within this syncytial cell must find a way to functionally compartmentalize their common cytoplasm in order to regulate nuclear division and symmetry breaking events. One way they solve this organizational problem is through the heterogeneous association of low-complexity sequence/polyQ-containing RNA-binding proteins with target RNAs. We have demonstrated that the proteins, Whi3 and Puf2, bind mRNAs to form aggregate structures that create sections of cytoplasm that are differentiated from one another. Further, we showed that assembly of these proteins with key cell cycle and cell polarity transcripts allow the cell to control the diffusivity of mRNA localization through space and time. We hypothesized that the multivalent interactions of these proteins with RNAs are sufficient to drive the assembly of phase-separated droplets that form non-membranous compartments within the cytoplasm. In support of this, cell-free reconstitution techniques along with structured illumination microscopy has revealed that these proteins form different structures whether in the presence of RNA, and that these structures can physically change over time depending on the local concentration of RNAs. Currently, we are investigating how the specificity and arrangement of the protein binding sites on the mRNAs can catalyze the coalescence of these aggregation-prone proteins through the use of cell-free assays, smRNA FISH, and single molecule pull down (SiMPull) for RNAs. Further investigation will focus on the role of

the local environment and cellular stress has on controlling assembly of these physiological RNA-protein complexes.

P237

Board Number: B349

Cyclin mRNA regulation by dynamic Whi3 RNA-protein droplets.

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In the multinucleate filamentous fungus *Ashbya gossypii*, many aspects of cellular growth and activity are spatially and temporally confined to local zones within a single, large cell. For example, nuclei that are only micrometers apart divide asynchronously despite being bathed in the same continuous cytosol where freely diffusing components would be predicted to be equal in all regions. This raises the problem as to how in *Ashbya* functional cytoplasmic compartments can be created that give rise to local territories with distinct activity from neighboring territories. One solution employed by the fungus is to create phase-separated droplets of protein and mRNA that are distributed non-homogeneously in the cell. Asynchronous nuclear division is dependent upon the non-uniform positioning of cyclin transcripts by Whi3, an RNA-binding protein with a low complexity sequence that allows it to undergo regulated aggregation. This work considers how Whi3 complexes are regulated by environmental conditions and how the complexes function to promote local production of the cyclin, Cln3, protein. Whi3 aggregates are known to respond to cellular stresses by changing their size and distribution in a manner specific to the environmental challenge. Single molecule FISH data will be presented to show how these changes in Whi3 aggregation correspond to changes in Cln3 transcript localization. The degree to which cyclin transcripts are protected or masked by Whi3 protein complexes so as to limit translation during different phases of the cell cycle or in response to environmental stress is also being investigated. This work uses protease digestion assays combined with single molecule FISH. These data provide insight into how multinucleate cells use functional RNA-protein aggregates to create dynamic compartments that organize the cytoplasm.

P238

Board Number: B350

How prolonged prometaphase blocks daughter cell proliferation despite normal completion of mitosis.

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We previously reported that when untransformed human (RPE1) cells spend >1.5 hr in prometaphase, their daughters uniformly exhibit a p38 dependent arrest in G1 despite normal division of their mothers. Transient inhibition of p38 activity allows daughters born of prolonged prometaphase to proceed

through interphase and mitosis of normal duration, yet all granddaughters arrest in G1 - suggesting a durable change in the properties of the daughter cells that cannot be erased by progression through interphase and mitosis. What happens during prolonged prometaphase and the nature of the change in daughter cell properties were unknown. Here we find that during prometaphase the progressive loss of activity of the anti-apoptotic protein MCL1 and oxidative stress at ambient oxygen levels can act independently or in concert to diminish the proliferative capacity of the daughters. Additional observations provide functional evidence that a slow activation of the apoptotic pathway participates in the initiation of the G1 arrest of daughter cells. Since some spindle defects prolong prometaphase but eventually allow mitotic checkpoint satisfaction, this phenomenon can prevent unresolved defects in mitosis from being propagated to future cell generations.

P239

Board Number: B351

Activation of Plk1 pool in late G2 promotes mitotic commitment.

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Activation of the master driver CyclinB1-Cdk1 in late G2 orchestrates the cell division program. How this trigger event is timely controlled during each cell cycle is still an open question. Here, we investigated upstream regulatory mechanisms focusing on the involvement of Polo-like kinase 1 (Plk1). Its activity is limiting for mitotic commitment during unperturbed cell cycles and we determined that activation of Plk1 pool is taking place in late G2 and reproducibly precedes Cyclin-B1-Cdk1 one by a few minutes. This sudden activation relies on upstream Aurora-A kinase and appears related to CyclinA2-Cdk1&2 activity level. In fact, stimulating CyclinA2-Cdk activity from late S, by inhibiting Wee1 kinase, induced a premature activation of Plk1 preceding unscheduled entry into mitosis. We determined that Plk1 associates with Cdc25C in interphase and that its activation induces concurrent Cdc25C phosphorylation before mitotic entry. Mimicking Plk1-dependent Cdc25C phosphorylation events, we found that this regulatory mechanism is sufficient to promote mitotic entry. Altogether, our results unravel some upstream molecular steps leading to CyclinB1-Cdk1 initial activation and mitotic entry.

P240**Board Number: B352****CATS (FAM64A) ALTERED EXPRESSION REDUCES PROLIFERATION OF HEMATOPOIETIC CELLS.**I. Barbutti¹, J.M. Xavier¹, S.T. Saad¹, L.F. Archangelo^{1,2};¹Hematology and Hemotherapy Center, University of Campinas, UNICAMP, Campinas, Brazil, ²University of São Paulo (USP) at Ribeirão Preto, Ribeirão Preto, Brazil

CATS (FAM64A) is a nuclear protein expressed according to the different cell cycle phases, and its expression peaks during S and G2/M. Moreover, a function in metaphase-anaphase transition has been attributed to FAM64A. The protein can be used as a marker for proliferation since its expression positively correlates with proliferation. The precise role of CATS (FAM64A) in proliferation and cell cycle control remains to be defined. The aim of this study was to investigate the effects of CATS (FAM64A) depletion and overexpression of hematopoietic cells. Lentivirus targeting CATS (FAM64A) silencing were used to transduce the U937 cells. Depletion was confirmed at RNA (qRT-PCR) and protein (Western blotting) levels. FAM64A depleted (shFAM64A) and control cells underwent functional *in vitro* assays and cell lysates were used for Western blotting analysis. Proliferation was assessed by MTT assay and colony forming unit (CFU), grown in semi-solid media without growth factors, and cell cycle assay was performed by FACS analysis of the DNA content. Overexpression of the murine Cats (Fam64a) was achieved by retrovirus transduction of primary bone marrow cells with pMIG-Fam64a. Successfully transduced cells were sorted for GFP expression, submitted to cell cycle analysis and cultivated in semi-solid media supplemented for myeloid differentiation. GM (granulocytes and monocytes), BFU-E (erythrocytes) and GEMM (progenitors) colonies and total number of cells were counted after 8 days. Cells were replated and colonies were counted after 10 days. FAM64A depletion was about 80% in shFAM64A cells. Cell proliferation was reduced in shFAM64A cells by 20% in liquid and by 66% in semi-solid media compared to control. Accordingly, a significant reduction of 12% of shFAM64A cells in S phase of the cell cycle was observed. The CYCLIN E and B1 expression were decreased in shFAM64A, whereas CYCLIN A and D1 expression were similar to control cells. Expression of *Fam64a* in mice cells was 215-fold higher in pMIG-Fam64a than in control cells. A significant reduction of GM (by 55%) and GEMM (by 78%) pMIG-Fam64a colonies was observed when compared to control, whereas GM pMIG-Fam64a colonies were reduced by 84% after replating. Total number of pMIG-Fam64A cells was also significantly reduced by 58%. Cell cycle analysis of transduced cells showed a significant reduction of 5% of cells in the S phase of the cell cycle in pMIG-Fam64a compared to control cells. Our results demonstrate that abnormal expression of FAM64A on hematopoietic cells leads to a decrease in cell proliferation which correlates with a decrease in percentage of cells in the S phase of the cell cycle. In human cells, this decrease also correlates with reduced CYCLIN E and B1 expression.

P241**Board Number: B353****CDK1-dependent Inhibition of the E3 Ubiquitin Ligase, CRL4CDT2, Ensures Robust Transition from S Phase to Mitosis.**L.F. Rizzardi¹, D. Varma¹, K.E. Coleman¹, J.P. Matson¹, S. Oh¹, J.G. Cook¹;¹Biochemistry and Biophysics, UNC Chapel Hill, Chapel Hill, NC

Replication-coupled destruction of a cohort of cell cycle proteins ensures efficient and precise genome duplication. Three proteins destroyed during replication via the CRL4CDT2 ubiquitin E3 ligase, CDT1, p21, and SET8 (PR-SET7), are also essential or important during mitosis making their re-accumulation after S phase a critical cell cycle event. During early and mid-S phase and during DNA repair, PCNA loading onto DNA (PCNADNA) triggers the interaction between CRL4CDT2 and its substrates. We have discovered that beginning in late S phase, PCNADNA is no longer sufficient to trigger CRL4CDT2-mediated degradation. A CDK1-dependent mechanism that blocks CRL4CDT2 activity by interfering with CDT2 recruitment to chromatin actively protects CRL4CDT2 substrates. We postulate that deliberate override of replication-coupled destruction allows anticipatory accumulation in late S phase; we further show that (as for CDT1) de novo SET8 re-accumulation is important for normal mitotic progression. In this manner, CDK1-dependent CRL4CDT2 inactivation contributes to efficient transition from S phase to mitosis.

P242**Board Number: B354****Diaphanous formin is required for epigenetic inheritance of CENP-A at centromeres.**C. Liu¹, Y. Mao¹;¹Department of Pathology and Cell Biology, Columbia University College of Physicians Surgeons, New York, NY

Epigenetic landscape has to be well inherited over cellular and organismal generations, largely independent of the underlying DNA sequences. In mammals, as the fundamental unit for chromosome segregation during mitosis, centromeres are defined epigenetically by nucleosomes containing the histone H3 variant CENP-A. In order to maintain centromere identity against CENP-A dilution as DNA duplicates and cell divides, newly synthesized CENP-A proteins are deposited at preexisting centromeres specifically during early G1 of each cell cycle. However, little is known about how accurate CENP-A inheritance is achieved. Recent efforts in the field have elucidated pathways involving different licensing, loading and stabilizing factors required for CENP-A deposition and maintenance, among which a small Rho GTPase molecular switch has been proposed to stabilize newly incorporated CENP-A at the centromere. The FH2 domain of mammalian diaphanous-related formin mDia2, which is a Rho family GTPase effector, has been shown to co-complex with various nucleosome proteins including histone H3

in mammalian cell proteomics (Daou et al. MBoC, 2014). Knocking down mDia2 resulted in substantially reduced levels of centromeric CENP-A revealed by quantitative imaging and high-throughput, unbiased measurement with fixed HeLa cells. Notably, this reduction was not caused by the loss of centromeres, as total number of CENP-B foci in knockdown cells was not significantly changed. A siRNA resistant construct of WT mDia2 rescued this phenotype by restoring CENP-A levels at centromeres. High resolution ratiometric live cell imaging (developed in this study) using HeLa cells stably expressing YFP-CENP-A, documented defective CENP-A loading over the 10 hour time window after anaphase onset in cells depleted of mDia2. Meanwhile, SNAP-tag pulse chase assay revealed that mDia2 is required specifically for newly synthesized CENP-A at centromeres. Furthermore, in mDia2 depleted cells, expression of actin mutants of mDia2 didn't restore CENP-A levels at centromeres. Therefore, our findings have uncovered a novel role for diaphanous formin in regulating CENP-A deposition and thus the epigenetic inheritance of centromere identity.

P243

Board Number: B355

Spatial regulation of mitotic timing.

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One of the most prominent characteristics of cyclin B1 is its dynamic localisation throughout the cell cycle. We, and others, have shown that cyclin B1 shuttles between the cytoplasm and the nucleus, but its greater rate of export over import ensures that the majority of cyclin B1 is cytoplasmic throughout interphase. At mitosis, cytoplasmic cyclin B1 is rapidly imported into the nucleus concomitant with the activation of its partner kinase, Cdk1. Although we understand the mechanism by which cyclin B1 is maintained in the cytoplasm, the function of this spatial distribution is still not understood. In order to study the function of cyclin B1 localisation throughout the cell cycle we made use of a cyclin B1 knock out mouse line. We have shown earlier that cyclin B1 null embryos arrest at the four-cell stage and is therefore the only truly essential cyclin in the mouse. This system allowed us to avoid the potential pitfalls of RNAi based approaches by injecting mRNAs encoding mutant forms of cyclin B1 into a genetically null background. Moreover, the large cell size of the early mouse embryo makes this system particularly well suited to study spatial controls using a 3D imaging approach. To increase throughput (as only 25% of embryos are B1 null in each experiment) we have developed a novel imaging chip that allows high resolution imaging of large numbers of mouse embryos as well as their retrieval for genotyping at the end of the experiment. Using this experimental setup we studied the role of nuclear export with respect to cell cycle timing by injecting constitutively nuclear cyclin B1 into B1 null embryos. We found that injected nuclear cyclin B1 can drive up to three division cycles. Furthermore, maintaining cyclin B1 in the nucleus had little effect on the duration of mitosis, nor did it appear to have any measurable effect on DNA replication, because both G1 and S phase durations were the same as in

embryos injected with wild type cyclin B1. Nuclear cyclin B1 did, however, significantly accelerate entry to mitosis after the end of DNA replication, as G2 phase was 40% shorter than normal. We have further tested the functionality of membrane tethered Cyclin B1 and found that it cannot drive entry into mitosis in a null background. Thus, the ability to export cyclin B1 into the cytoplasm is important for cells to regulate the correct timing of mitosis.

P244

Board Number: B356

Cdk4/6-dependent activation of FOXM1 controls recovery from DNA damage in G1.

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Most Cyclin-dependent kinases are redundant for normal cell division. We tested whether these redundancies are maintained in the abnormal situation of cell cycle reentry after a DNA damage-induced arrest in G1. Interestingly, using non-transformed RPE and BJ-Tert cells, we find that while Cdk4 is dispensable both for S-phase entry of normally proliferating cells and for cell cycle re-entry after serum starvation, it becomes essential to drive S-phase entry after irradiation. Overexpression of Cdk6 can overcome this dependence on Cdk4, indicating that the overall level of Cdk4/6 activity becomes rate-limiting during recovery. We show that Cdk4/6 kinase activity is only required once the checkpoint has been turned off, suggesting that Cdk4/6 activity is not required for DNA repair or checkpoint silencing. Indeed, DNA repair and the elimination of Cdk inhibitor proteins p21 and p27 are unaffected after depletion of Cdk4. We find that Cdk4/6 activity is required to phosphorylate pRb after irradiation, yet inactivation of pocket proteins is insufficient to allow S-phase entry after DNA damage when Cdk4 is depleted. Instead, our data demonstrate that Cdk4 drives recovery from a G1 arrest through the activation of FOXM1.

P245

Board Number: B357

Two sequential decisions commit human cells to start the cell cycle.

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One of the most fundamental decision processes in biology is the commitment of cells to enter the cell cycle and generate two daughter cells. Cell-cycle commitment in mammalian cells is thought to be regulated by a single decision point in G1 called the Restriction Point, after which growth factors are no longer required for the cell cycle to proceed. Using live-cell microscopy of cell-cycle sensors and single-

cell analysis, we have identified the moment at which cells cross the Restriction Point and find that more than three hours pass between the Restriction Point and the actual start of DNA replication. Furthermore, we find that cell stress after the Restriction Point but before the start of DNA replication results in cell-cycle exit and a return to a pre-Restriction Point state, providing evidence for another later decision to commit to the cell cycle. We present evidence for a second bistable switch regulating S-phase entry. The trigger for this second switch is the CDK2/Cyclin E-mediated partial inactivation of APCCdh1, an E3-ligase that controls the degradation of proteins necessary for S-phase progression. We show that the switch is driven by a double negative feedback between the regulatory protein Emi1 and APCCdh1. Markedly, Emi1 functions as both an inhibitor and a substrate of the APC, which generates a bistable and irreversible switch regulating S-phase entry. Thus, cells commit to the cell cycle by making two sequential decisions. They first monitor mitogen signals to decide whether to cross the Restriction Point, and then monitor stress signals to decide whether to irreversibly inactivate APCCdh1, start DNA replication, and fully commit to the cell cycle.

P246

Board Number: B358

hNek5 interferes negatively in mitochondrial mediated apoptosis and respiration.

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Beside to supplying cellular energy, mitochondrias are involved in signaling, cell death and cellular differentiation and have been implicated in several human diseases. Neks (NIMA-related kinases) represent a family of mammals protein kinases that plays essential roles in cell-cycle progression, but other functions are recently related. Nek5 is the kinase with the smallest information. To characterize Nek5 functions, the yeast two-hybrid (Y2H) screening was performed. According to GO, mitochondrial proteins were identified as hNek5 partners. The mitochondria location of hNek5 was confirmed by cell fractionation followed by western blot analysis and colocalization between hNek5 and mitochondrial proteins in U2-OS and MCF-7 by confocal microscopy. As the prey are involved on apoptosis, mitochondrial transport and respiratory chain complex assembly, the involvement of hNek5 in the mitochondria context was analyzed by MTS and annexin V-FITC assay, measurement of ROS generation and the oxygen consumption. Apoptosis assay showed protective effects of hNek5 in Hek293-T's cell death after thapsigargin treatment (2uM). Stable cells expressing hNek5 or the kinase dead (K33A) version were generated using the FLP-In T-REX mammalian expression system and shRNA lentiviral particles were used to silence hNek5. Silenced cells as well as kinase dead displayed an increase on ROS formation after 4 hours of thapsigargin (2uM) treatment, but, the potentiation effects on cells death occur only after 16 hours of thapsigargin treatment. Cells expressing hNek5, showed protective effects on cell death and ROS formation. Mitochondrial respiratory chain activity of stable and depleted hNek5 cells was determined using the Oroboros Oxygraph. Mitochondrial respiratory chain activity is impaired in the presence of hNek5. Cells silenced for hNek5 presented rampant rates of electrons transfer from

ascorbate to cytochrome c and in the complex II. The partners identified by Y2H are potential substrates for hNek5 that mediates the defects on respiratory chain. In conclusion our data suggest for the first time the hNek5 mitochondrial localization and its role on cell death and cell respiration defects.

Oncogenes and Tumor Suppressors 1

P1837

Board Number: B360

Involvement of calreticulin in α -integrin mediated survival and chemoresistance in T-cells.

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Cell adhesion mediated drug resistance (CAM-DR) is a major contributor to relapse in hematological malignancies. Previously, we showed that adhesion via $\alpha 4\beta 1$ or $\alpha 5\beta 1$ integrins promotes chemoresistance in Jurkat T-leukemia cells (Mol Cell Biol 33:4334). Likewise, adhesion via other integrins including $\alpha 2\beta 1$ or $\alpha L\beta 2$ also promotes chemoresistance, pointing to a common conserved signaling mechanism. The membrane proximal KxGFFKR motif is highly conserved in the cytoplasmic domain of all α -integrins. Expression of a non-integrin chimeric receptor with only KxGFFKR as the cytosolic motif rendered cells highly chemoresistant in an adhesion-independent manner. These cells exhibit high rates of calcium influx coupled with drug efflux, as well as constitutive activation of Akt pro-survival signaling. Furthermore, we found increased interaction of KxGFFKR with the calcium-binding protein, calreticulin (CRT). Recently, high frequency somatic mutations that give rise to a variant CRT protein predicted to be enriched in the cytosol (vs the endoplasmic reticulum) was implicated in the JAK-STAT pathway controlling myelo- and lympho-proliferative disorders. As CRT-integrin association can be stimulated by cell adhesion, we propose that cytosolic CRT may act as an oncogenic protein involved in cell survival and regulation of chemoresistance.

To investigate the role of CRT in CAM-DR, we generated CRT null T-cells by CRISPR-Cas mediated gene silencing, and show that CRT is implicated in integrin activation, cell adhesion, and enhanced apoptotic resistance to cytotoxic chemotherapy. Furthermore, interferon-stimulated phosphorylation of STAT3 and STAT5 is disrupted in CRT null T-cells, suggesting CRT has a vital role in coupling cytokine-mediated stimulation of JAK-STAT signaling in lymphoblasts. Ongoing investigation will further delineate the cytosol and endoplasmic reticulum specific roles of CRT in CAM-DR by intracellular targeted CRT expression enriched in the cytosol and/or ER.

P1838**Board Number: B361****iRHOM2 regulation of ADAM17 as a key regulator of epithelial growth factor signalling.**M.A. Brooke¹, S.L. Etheridge¹, B. Fell¹, D.P. Kelsell¹;¹Blizard Institute, Barts and The London School of Medicine Dentistry, London, United Kingdom

Our group has previously linked point mutations in the proteolytically inactive rhomboid protein iRHOM2 to the dominantly-inherited disease Tylosis with oesophageal Cancer (TOC), a disorder of palmo-plantar keratoderma, oral leukokeratosis, and the only known syndrome of inherited oesophageal squamous cell carcinoma susceptibility. In keratinocyte cell lines, these iRHOM2 mutations result in dysregulation of the multi-substrate ectodomain sheddase enzyme ADAM17 (TACE), an enzyme whose maturation and activation is regulated by iRHOM2.

TOC-associated iRHOM2 mutations result in increased ADAM17 maturation and translocation to the plasma membrane of keratinocytes (in both monolayer and three-dimensional epidermal-equivalent cultures), and greatly upregulated constitutive shedding of numerous ADAM17 substrates, including the EGF-family growth factors amphiregulin, TGF α and HB-EGF. This is accompanied by a phenotype of significantly increased proliferation and migration of TOC-associated iRHOM2-mutant cells relative to controls, with both the increased levels of growth factor shedding and the pro-migratory phenotype sensitive to pharmacological ADAM17 inhibition or siRNA knockdown. Excessive EGF-family growth factor signalling was also shown to result in the upregulation of Transglutaminase 1 activity in the epidermis of TOC-affected individuals and in three-dimensional TOC keratinocyte cultures relative to controls, whilst the epidermis of TOC-affected individuals is also characterised by the expression of desmosomes lacking electron-dense midlines, suggestive of the presence of a pro-migratory, wound-healing-like state in the skin. The presence of a constitutive wound-healing-like state is also supported by the very strong constitutive upregulation of IL-6 and IL-8 expression and secretion by TOC keratinocytes, secretion which is sensitive to ADAM17 knockdown despite these cytokines not being ADAM17 substrates.

In addition to regulation of EGF-family growth factor shedding, iRHOM2 may also play a role in the transactivation of EGFR signalling in response to G-protein coupled receptor (GPCR) activation, a process dependent on ADAM17 activity. Activation of the GPCR FGFR2b on keratinocytes by the growth factor KGF leads to phosphorylation of iRHOM2 and ADAM17, suggesting that activation of iRHOM2, and the interaction between it and ADAM17 may play an important role in regulating EGFR transactivation in response to GPCR signalling.

These findings reveal the central role of iRHOM2 in growth factor regulation in the skin. This, and the association of iRHOM2 mutations with oesophageal cancer may offer new insights into mechanisms of epidermal hyperproliferation and oesophageal carcinogenesis.

P1841**Board Number: B362****Pro-apoptotic role of human YPEL5, yeast ortholog MOH1, in DNA damage-induced apoptosis in tumor cells.**J. Lee¹, D. Jun², L. Xie¹, Y. Kim¹;¹School of Life Science and Biotechnology, College of Natural Sciences, Kyungpook National University, Daegu, Korea, ²Institute of Life Science and Biotechnology, Kyungpook National University, Daegu, Korea

During comprehensive sequence analysis of human chromosome, a novel gene family consisting of five members (YPEL1 through YPEL5) showed high conservation among eukaryotes from yeast to human. To understand the functional role of YPEL5, which is distinct from the other four members, we employed the budding yeast (*Saccharomyces cerevisiae*) mutant possessing disrupted yeast ortholog MOH1 gene, and compared its physiological characteristics with those of the wild-type strain. The mutant for the MOH1 gene exhibited an enhanced resistance to UVC (254 nm) irradiation and genotoxic agents as compared with the wild-type. This phenomenon was abrogated when the disrupted MOH1 gene in the mutant was restored by transformation of the human YPEL5 gene. In tumor cells, the level of YPEL5 was up-regulated following treatment with genotoxic agents and the up-regulation of YPEL5 at the transcription level appeared to depend on the tumor suppressor p53. Whereas the overexpression of YPEL5 augmented DNA damage agent-induced caspase cascade activation, the down-regulation of YPEL5 could suppress the apoptotic events. These results demonstrate the functional conservation of YPEL5 protein between lower and higher eukaryotes, which is involved in DNA damage-induced apoptosis, and provide insights into the molecular and cellular function of the YPEL family proteins.

P1842**Board Number: B363****Mechanisms of p53-dependent Cell Fate Choice.**K.D. Sullivan¹, J.M. Espinosa¹;¹HHMI and MCDB Department, University of Colorado, Boulder, CO

The p53 tumor suppressor governs cellular stress responses ranging from cell cycle arrest to apoptosis; however, the molecular mechanisms of these cell fate decisions remain poorly understood. Attempts at small molecule activation of p53 have fallen short clinically as they result in reversible cell cycle arrest in the majority of cancer cell types. We have performed genome-wide shRNA screens with one such small molecule, Nutlin-3, to identify genes that are lethal with non-genotoxic activation of p53 and discovered that the ATM and MET kinases regulate p53-dependent cell fate choice. Genetic or pharmacological interference with ATM or MET converts the cellular response to Nutlin-3 from cell cycle arrest to apoptosis. Nutlin-3 and ATM synthetic lethality owes to functions of ATM outside the DNA damage response. We show that inhibition of ATM results in accumulation of intracellular reactive oxygen species (ROS), and that these ROS are involved in the synthetic lethality as the phenotype is abrogated by addition of the ROS scavenger N-acetyl-cysteine. Furthermore, knockdown of several p53-inducible

antioxidant genes including SESN1/2 and TP53INP1 further sensitizes cells to Nutlin-3 and ATM synthetic lethality. We demonstrate that ROS increases associated with a loss in ATM activity correlate with accumulation of mitochondria and provide evidence that this may be linked to defects in autophagy. Finally, to identify genes that are required for Nutlin-3 and ATM induced synthetic lethality; we performed a positive selection screen that identified several promising candidates of diverse biological function. Further studies are also under way to better understand the conservation of synthetic lethality across cancer types and between drugs with different mechanisms of action.

P1845

Board Number: B364

Profiling the Molecular changes during malignant transformation and response to different oxygen levels, using a combined transcriptomics and proteomics approach.

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Transformation of normal cells to cancer cells that are able to form tumors and even metastasize is a multi step process where molecular changes of high complexity are acquired. By using a four-stage isogenic human cell model that mimics the route to malignancy we demonstrate how a transcriptomics approach with subsequent protein analysis can be used to define the molecular changes that accompany the mechanisms related to immortalization, transformation and invasion/metastasis separately and identify potential biomarkers for malignancy. Additionally, we are currently investigating the impacts of hypoxia by studying differential protein expression and spatial distributions of proteins under different oxygen levels. This project is performed in close collaboration with the Human Protein Atlas (HPA) project and with the use of RNA sequencing in combination with the HPA collection of antibodies covering the whole human proteome and high-resolution microscopy, protein expression can be studied on a single cell level. Our results show that about 1300 genes are differentially expressed across the cell model and that the majority of the genes are downregulated, supporting the principle of dedifferentiation en route to malignancy. [1]

1. Danielsson, F., et al., Majority of differentially expressed genes are down-regulated during malignant transformation in a four-stage model. Proceedings of the National Academy of Sciences of the United States of America, 2013. 110(17): p. 6853-8.

P1846**Board Number: B365****p21-activated kinase 4 promotes breast cancer initiation and progression.**

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p21-activated kinase 4 (PAK4) is overexpressed in diverse human cancers and plays a pivotal role in several cancer-associated cellular events.

To elucidate the role of PAK4 in breast cancer initiation and progression *in vivo*, we have generated transgenic mice that overexpress PAK4 in the mammary gland (MMTV-PAK4). MMTV-PAK4 mice exhibit atypical hyperplasias that eventually progress to cancer, indicating that PAK4 overexpression in the mammary gland is sufficient *per se* to increase the spontaneous mammary carcinoma incidence.

Conversely, we have also established mice with conditional PAK4 depletion in the mammary epithelium (PAK4 floxed;MMTV-Cre). Conditional knockout of PAK4 significantly delays PyMT-driven mammary tumourigenesis. Consistently, ablation of PAK4 inhibited human breast cancer xenograft growth in mice.

Through the combination of several global approaches (including exome sequencing to identify genetic events that accompany PAK4-driven tumourigenesis and global expression profiling of cancer cells upon PAK4 inhibition) we aim to gain a comprehensive insight into how PAK4 regulates breast cancer initiation and progression.

P1848**Board Number: B366****Characterization of the SW13 adrenal adenocarcinoma cell line and induction of subtype switching by HDAC1 inhibition.**

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The human adrenal carcinoma SW13 cell line can switch between a subtype which expresses the tumor-suppressor gene Brahma (Brm), SW13+, and one that does not, SW13-. Unlike many tumor suppressor genes, which are often mutated, in SW13- cells loss of Brm expression occurs post-transcriptionally and can be restored via histone deacetylase (HDAC) inhibition. However, many of the previously characterized Brm inducing HDAC inhibitors are toxic, broad-spectrum inhibitors which have limited downstream use, and provide little insight into the specificity of epigenetic Brm suppression. In this work we sought to further characterize the oncogenic potential of the two SW13 subtypes, and to

investigate the potential epigenetic regulators that contribute to the subtype switching. Cell growth and colony formation assays revealed that Brm deficient SW13- cells have both a faster cell number doubling time and an increased rate of anchorage-independent growth, while SW13+ cells appear to express higher levels of MMP. Next, we screened multiple HDAC inhibitors for their ability to induce switching between the SW13 subtypes in an effort to identify the specific HDAC enzyme(s) necessary for this conversion. Immunofluorescence and gene expression analyses suggest that inhibition of HDAC1 most efficiently increases the rate of conversion between SW13- and SW13+ cells, and that it does so in a dose dependent manner. Furthermore, after conversion to SW13+ there is increased acetylation on histone H3 but not histone H4 when compared to levels in SW13-, suggesting that the role of acetylation on the H3 tail may play an essential role in the subtype switch. Pathway-specific quantitative RT-PCR profiling arrays uncovered significant differences in the expression of multiple genes involved in epigenetic chromatin remodeling as well as epigenetic chromatin modification, indicating that these two subtypes also have unique gene expression profiles that could contribute to Brm regulation and maintenance of the subtype switch. These studies provide new insight into the functional consequences of Brm loss in the SW13 cell type and reveal potential targets for the reversal of Brm epigenetic silencing.

P1851

Board Number: B367

Proteomic analysis of EWS-Fli-1 associated proteins shows the contribution of lysosomes in EWS-Fli-1 turnover.

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Ewing sarcoma is an aggressive pediatric cancer of the bone and soft tissues characterized by a chromosomal translocation involving EWS and an Ets transcription factor, usually Fli-1. EWS-Fli-1 fusions comprise 85% of Ewing sarcoma cases. Ewing sarcoma cells are dependent upon EWS-Fli-1 for growth and survival, yet little is known about EWS-Fli-1's biochemical characteristics. Using a proximity-dependent biotinylation technique, BioID, we identified cation-independent mannose 6-phosphate receptor (CIMPR) as a protein located in the vicinity of EWS-Fli-1. CIMPR is a cargo transporter which facilitates the movement of lysosomal hydrolases between the trans-Golgi network and the endosome, which are then transferred to the lysosome. We verified the interaction of CIMPR with EWS-Fli-1 by co-immunoprecipitation and discovered the role of lysosomes in regulating EWS-Fli-1 stability. Inhibition of lysosome function by chloroquine stabilized EWS-Fli-1 protein levels, while inhibition of the mTORC1 pathway by torin 1, which activates the TFEB-lysosome pathway, degraded EWS-Fli-1. These results suggest that lysosomal activation may provide a therapeutic means to modulate EWS-Fli-1 levels.

P1852**Board Number: B368****Hypersensitivity to DNA double strand breaks in late G2 protects against checkpoint adaptation.**L. Krenning¹, F.M. Feringa¹, R.H. Medema¹;¹Division of Cell Biology, the Netherlands Cancer Institute, Amsterdam, Netherlands

The cellular response to DNA damage ranges from transient cell cycle arrest to the induction of apoptosis or senescence. It is generally thought that permanent cell cycle withdrawal is initiated in response to severe, irreparable DNA damage. This way cells can prevent the propagation of a damaged genome. Alternatively, some cells may enter mitosis in the presence of DNA damage, a process known as checkpoint adaptation. This is a potentially dangerous process, as the propagation of damaged DNA may compromise genome integrity. Using live-cell microscopy to study the effects of DNA damage on cell fate decisions we have identified that a fraction of cycling cells are hypersensitive to DNA double strand breaks. This fraction represents cells that are damaged just prior to mitotic entry. These cells respond to DNA damage by activating the anaphase promoting complex / cyclosome in complex with its coactivator Cdh1 (APC/CCdh1) and undergo permanent cell-cycle exit, even in response to low amounts of DSBs. During unperturbed G2 progression the APC/CCdh1 is inhibited by Early mitotic inhibitor (Emi1)-binding to Cdh1, and CDK-dependent phosphorylation of Cdh1. However, as Emi1 levels decline towards the later stages of G2, these cells become critically dependent on CDK activity for APC/CCdh1-inhibition. Indeed, inhibiting CDKs in unstressed cells is sufficient to induce APC/CCdh1-activation specifically in late G2 cells. Conversely, preventing DNA damage-induced CDK inhibition prevents APC/CCdh1 activation in late G2 cells. In addition, overexpression of Emi1 prevents the DNA damage induced activation of APC/CCdh1. We continue to show that this cell cycle exit mechanism functions to prevent late G2 cells from entering mitosis in the presence of DNA double strand breaks, and therefore acts to prevent checkpoint adaptation and guard genome stability.

P1855**Board Number: B369****The role of RhoGAPs in basal-like breast cancer.**C.D. Lawson^{1,2}, K.L. Rossman², C. Fan³, C.M. Perou³, K. Burrige¹, C.J. Der²;¹Department of Cell Biology and Physiology, University of North Carolina at Chapel Hill, Chapel Hill, NC,²Department of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC, ³Department of Genetics, University of North Carolina at Chapel Hill, Chapel Hill, NC

The basal-like subtype of human breast cancer accounts for a disproportionately high percentage of overall breast cancer recurrence and death, and the current therapeutic options for this cancer are ineffective. Hence, elucidating the signaling pathways that are responsible for driving the growth of basal-like tumors may identify novel targets for the development of effective therapies. Rho family small GTPases have been implicated previously in promoting tumor cell proliferation, invasion, and metastatic

growth in a variety of cancers. These proteins are activated by guanine nucleotide exchange factors (GEFs) and, in the context of cancer, overexpressed RhoGEFs can function as oncogenes which cause hyper-elevated Rho GTPase activity. In contrast, GTPase-activating proteins (GAPs), which return Rho GTPases to an inactive, GDP-bound state, have generally been presumed to act as tumor suppressors. Surprisingly, microarray analysis of the expression of Rho GTPases, GEFs, and GAPs across a panel of human breast tumors revealed that a number of RhoGAP genes were significantly upregulated in basal-like breast cancers (BLBCs). These preliminary results suggest that RhoGAPs may play an unexpected role in promoting tumor growth. The aim of our research is therefore to validate and characterize the role of two of these RhoGAPs, RacGAP1 (also known as MgcRacGAP and CYK4) and ArhGAP11A (also known as MP-GAP), in BLBC development. In human BLBC cell lines, shRNA-mediated knockdown of either of these genes resulted in a significant defect in proliferation relative to control cells, in addition to distinct morphological changes. Furthermore, knockdown of RacGAP1 caused an increase in the number of multinucleated cells, consistent with a previously-established role for this Rac-specific GAP in regulating cytokinesis. In contrast, ArhGAP11A, a relatively poorly-characterized Rho-specific GAP, caused BLBC cell lines to accumulate in the G1 phase of the cell cycle upon its knockdown, indicative of a role in regulating cell cycle progression. In summary, we have established that two RhoGAPs, RacGAP1 and ArhGAP11A, are critical to the growth of BLBC cell lines. Experiments to establish the precise mechanisms through which these GAPs elicit each phenotype are ongoing. This work was supported by the U.S. Army Medical Research and Materiel Command under Award No. W81XWH-14-1-0033.

P1856

Board Number: B370

Epigenetic silencing of MARVELD1 gene attenuates nonsense-mediated mRNA decay in lung cancer and represents a potential biomarker for detection of malignancy.

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Nonsense-mediated mRNA decay (NMD) pathway, a well-known mRNA quality control system, rapidly degrades mRNAs with harboring translation premature termination codons (PTC) in post-transcriptional process. Recent studies suggest that NMD regulation might implicate in tumorigenesis; however, the mechanism is poorly defined. Here, we found that novel nuclear factor MARVELD1 and the epigenetic modification of MARVELD1 promoter are involved in NMD regulation. We demonstrated that the expression of MARVELD1 gene is down-regulated in lung cancer, especially in small cell lung cancer. The reduced MARVELD1 level in lung cancer is due to its promoter hypermethylation. Treatment with DNA methyltransferase inhibitor can restore MARVELD1 expression. In addition, MARVELD1 physically interacts with NMD factor SMG1 and NMD target PTC-mRNA. The decreased MARVELD1 expression in lung cancer reduces NMD efficiency through diminishing the association of NMD complex component UPF1/SMG1 with PTC-mRNA. These results suggest that the epigenetic modification of MARVELD1 promoter links with the regulatory mechanism of NMD pathway in lung cancer cells.

P1858**Board Number: B371****Identifying the c-MET phosphorylation site regulated by CD82 in prostate tumor cells.**V. Janardan¹, J. Shady¹;¹Biomedical Sciences; Cell and Molecular Biology, Grand Valley State University, Allendale, MI

CD82 (KAI), a metastasis tumor suppressor protein is under-expressed in prostate as well as several other types of metastatic cancers. It inhibits cancer metastasis, but the mechanism through which regulation happens remains unclear. Various pathways are being explored in this lab, including regulation of c-MET, a growth factor receptor observed to have increased activation in tumor cells.

CD82 and c-MET do not co-localize, suggesting that CD82 indirectly downregulates c-MET. To be expressed, c-MET first needs to bind to its ligand, HGF. This growth factor encourages phosphorylation of c-MET, consequently activating it. C-Met has four tyrosine phosphorylation sites that include p-Tyr 1003, p-Tyr 1234/1235, p-Tyr 1349 and p-Tyr 1365. Knowing how each phosphorylation site of c-Met affects downstream signaling event, our lab is focused in identifying which site is regulated by CD82. Another tetraspanin CD151, which promotes tumor progression and metastasis, has been shown to associate with c-Met and the integrins, which CD82 associates with. We are currently exploring the levels and expression of CD151 and its association with c-Met in the presence and absence of CD82. This we believe will provide additional insight into how CD82 overall regulates c-Met and prevents prostate tumor metastasis.

P1859**Board Number: B372****p53 protein reduces centrosome amplification in human cells exposed to subtoxic levels of 2-chloroethyl ethylsulfide (2-CEES).**J.D. Fulkerson¹, E. Behrens², A. Zinn^{2,3}, C. Duncheon², T.J. Lamkin⁴, R.A. Bennett⁵;¹Biology, University of Southern Indiana, Evansville, IN, ²University of Southern Indiana, Evansville, IN,³Biological Sciences, University of Toledo, Toledo, OH, ⁴Molecular Signatures Section - Molecular Genetics, Air Force Research Laboratory, Wright-Patterson AFB, OH, ⁵Department of Biology, University of Southern Indiana, Evansville, IN

Mustard gas is a simple molecule that was first used as a chemical weapon in World War I. It is a powerful vesicant and alkylating agent that causes painful blisters on epithelial surfaces and increases the incidence of cancer in those exposed. The mechanism of mustard gas toxicity and tumorigenesis is not well understood but is thought to be mediated by its ability to induce oxidative stress and DNA damage. Interestingly, several proteins (including p53) that have been shown to either be targets of mustard gas or mediate mustard gas toxicity have also been shown to regulate centrosome duplication.

Centrosomes are small, non-membrane bound organelles that direct the segregation of chromosomes during mitosis through the formation of the bipolar mitotic spindle. Cells with more or less than two centrosomes during mitosis can segregate their chromosomes unequally, resulting in chromosome instability, a common phenotype of cancer cells. In our studies, we show that subtoxic levels of a mustard gas analog, 2-chloroethyl ethylsulfide (2-CEES), induces centrosome amplification and chromosome instability (CIN) in cells lacking p53. Additionally, we show that the reintroduction of p53 protein to cells exposed to subtoxic levels of 2-chloroethyl ethylsulfide (2-CEES) reduces centrosome amplification in, which may retard the mutation rate necessary for tumorigenesis. These data may support previous demonstrations of mustard gas toxicity targeting p53.

P1862

Board Number: B373

BRG1 Promotes Breast Cancer by Reprogramming Lipid Biosynthesis.

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Epigenetic mechanisms can drive the development and progression of cancer. Epigenetic changes in tumor cells hold particular promise as therapeutic targets because, unlike gene mutations, they are reversible. In one well characterized epigenetic mechanism the SWI/SNF enzymes control gene expression through their ATP-dependent remodeling of chromatin structure. We found that the levels of BRG1, one of two mutually exclusive SWI/SNF ATPases, were up-regulated in human and mouse primary mammary tumors. Reduction of BRG1 expression decreased cancer cell proliferation and tumorigenesis in a panel of breast cancer cell lines *in vitro*, and in MDA-MB-231 xenografts. Depletion of BRG1 reprogrammed cancer cell metabolism, decreasing DNA, RNA and lipid synthesis. BRG1 controlled *de novo* fatty acid synthesis via transcriptional regulation of key lipogenic enzymes, including Acetyl-CoA Carboxylase, Fatty Acid Synthase, and ATP Citrate Lyase. We propose that targeting BRG1 could reverse the higher proliferation-supporting rates of *de novo* fatty acid synthesis observed in tumors even when exogenous fatty acids are abundant. In support of this idea, reduction of BRG1 sensitized breast cancer cells to chemotherapeutic drugs used clinically. Therefore, BRG1 may be a novel and promising target for the epigenetic treatment of breast cancer.

P1863**Board Number: B374****Is Hsp90 inhibitor efficacy dependent on Cullin 5 expression?.**S. Wicks¹, G. Heck¹, T. Atuobi¹, E. Ehrlich¹;¹Biological Sciences, Towson University, Baltimore, MD

Cancer is the second leading cause of death in the United States. Despite considerable progress in development of new cancer therapeutics, over half a million people are expected to die from cancer this year. Hsp90 inhibitors target Hsp90, a chaperone whose clients include oncogenes that are overexpressed in many different cancers. Inhibition of Hsp90 results in proteasomal degradation of clients and inhibition of related signaling pathways in cancer cells. Despite its promise, Hsp90 inhibitors have shown little efficacy in clinical trials. Cullin 5 (Cul5), a ubiquitin ligase involved in regulation of Hsp90 clients, has been shown to be down-regulated in breast cancer cells suggesting that variation in Cul5 gene expression may be negatively impacting Hsp90 inhibitor efficacy. We have previously reported that Cul5 is required for the geldanamycin-induced degradation of two clinically relevant Hsp90 clients, ErbB2 and Hif1a. Through analysis of gene expression data from 62 cases of breast invasive carcinoma obtained from The Cancer Genome Atlas, we observed decreased expression of Cul5 in tumor compared to normal tissue in all but 11 patients ($t = -5.6155$, $df = 61$, $p\text{-value} = 5.119e-07$). Here we demonstrate that Cul5 expression correlates with sensitivity to 17-AAG. Downregulation of Cul5 expression in AU565 cells resulted in complete resistance to 17-AAG, where as control cells exhibited almost 80% cytotoxicity. Sensitivity to 17-AAG correlated with Cul5 transcript levels in breast cancer cell lines. MCF-7 cells expressed Cul5 at levels two-fold higher than MDA-MB-468 cells and were more than three times as sensitive to killing by 17-AAG. This data suggests that variation in Cul5 expression may be responsible, in part, for the variation in sensitivity to Hsp90 inhibitors observed in the clinic. By establishing Cul5 as a critical player in the response to Hsp90 inhibitors, we can study Cul5 downregulation in cancer with the goal of increasing Hsp90 inhibitor efficacy, thereby enhancing survival and quality of life for affected individuals.

P1866**Board Number: B375****CDKN3 plays an important role in cellular transformation by Abl oncogenes.**J. Chen¹, K. Chen¹, G. Guo¹;¹Chinese Academy Scis-Inst Microbiol, Beijing, China

Previous studies have revealed that deregulation of multiple signaling pathways associated with cell survival and proliferation, including phosphoinositide-3-kinase (PI3K)/AKT, RAS, and Janus kinase (JAK)/signal transducer and activator of transcription (STAT), underlies Abl oncogene induced tumorigenesis. However, the precise mechanisms by which Bcr-Abl causes leukemogenesis are not fully clarified. CDKN3 (cyclin-dependent kinase inhibitor 3), a dual specificity protein phosphatase, dephosphorylates cyclin-dependent kinases (CDKs) and thus functions as a key negative regulator of cell

cycle progression. Deregulation or mutations of CDKN3 have been shown to play roles in various cancers. However, the function of CDKN3 in Bcr-Abl-mediated chronic myelogenous leukemia (CML) remains unknown. Here we found that CDKN3 acts as a key inhibitor in Bcr-Abl-mediated leukemogenesis. Overexpression of CDKN3 sensitized the K562 leukemic cells to imatinib-induced apoptosis and dramatically inhibited K562 xenografted tumor growth in nude mouse model. Forced expression of CDKN3 significantly reduced the efficiency of Bcr-Abl-mediated transformation of FDCP1 cells to growth factor independence. In contrast, depletion of CDKN3 expression conferred resistance to imatinib-induced apoptosis in the leukemic cells and accelerated the growth of xenograph leukemia in mice. In addition, we found that CDKN3 mutant (CDKN3-C140S) devoid of the phosphatase activity failed to affect the K562 leukemic cell survival and xenografted tumor growth, suggesting that the phosphatase of CDKN3 was required for its tumor suppressor function. Furthermore, we observed that overexpression of CDKN3 reduced the leukemic cell survival by dephosphorylating CDK2, thereby inhibiting CDK2-dependent XIAP expression. Moreover, overexpression of CDKN3 delayed G1/S transition in K562 leukemic cells. Our results highlight the importance of CDKN3 in Bcr-Abl-mediated leukemogenesis, and provide new insights into diagnostics and therapeutics of the leukemia.

P1867

Board Number: B376

Inhibitors of Skp2 E3/Cks1 ligase complex prevent degradation of p27kip1 as a novel therapeutic approach to estrogen-induced type I endometrial cancer.

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In many human cancers, the tumor suppressor, p27kip1 (p27), a cyclin-dependent kinase inhibitor critical to cell cycle arrest, undergoes perpetual ubiquitin-mediated proteasomal degradation by the E3 ligase complex SCF-Skp2/Cks1 and/or cytoplasmic mislocalization; lack of nuclear p27 causes aberrant cell cycle progression and cytoplasmic p27 mediates cell migration/metastasis. We previously showed that estrogen (E2) induces Skp2-dependent degradation of p27 and cell proliferation in primary endometrial epithelial cells (EECs) and endometrial carcinoma (ECA) cell lines (e.g., ECC-1) suggesting a pathogenic mechanism for type I endometrial carcinoma (ECA), an estrogen (E2)-linked cancer. The current studies show that treatment of ECC-1 cells with small molecule inhibitors of Skp2/Cks1 E3 ligase activity (Skp2E3LIs) increase protein levels of p27, increase p27 half-life by 6 hours and inhibit cell proliferation (IC50 14µM) by G1 phase block and not by apoptosis. Two of five SKP2E3LIs, C2 and C20 specifically increase p27 in the nucleus by 2-fold while decreasing p27 in the cytoplasm by 33% in 18 hours in both ECC-1 cells and primary ECA cells. Similarly, by Super Resolution Microscopy, Skp2E3LIs increased nuclear p27 by 1.8-fold. In addition, C2 and C20 prevent both E2-induced proliferation and degradation of nuclear p27. Importantly, C2, C5, and N1 injected into ovariectomized, E2-primed mice increase nuclear p27 in uterine epithelial cells with a concomitant 42-62% decrease in proliferation. Taken together, these data suggest that Skp2E3LIs function in the nucleus to prevent E2-induced

degradation of p27 to regain growth control by directly blocking p27 ubiquitylation and subsequent degradation suggesting that these inhibitors have potential to prevent ECA since E2-induced hyperplasia precedes carcinoma. Skp2E3LIs enable a chemical biology approach to understanding the functional significance of the ubiquitin pathway in p27-mediated dysregulation of the cell cycle in cancer, and importantly, are the first specific proteasome inhibitors that pharmacologically target the binding interaction between the E3ligase, SCF-Skp2/Cks1 and p27 to critically stabilize nuclear p27 and prevent cell cycle progression. These targeted inhibitors represent a major therapeutic advancement over general proteasome inhibitors for cancers characterized by SCF-Skp2/Cks1-mediated destruction of p27.

P1870

Board Number: B377

Molecular and cellular mechanisms of Pin1-mediated Grb7 degradation in regulation of cancer progression.

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Growth factor receptor bound protein-7 (Grb7) is a multi-domain adaptor protein in cooperation with numerous tyrosine kinases to regulate various cellular signaling and functions. Indeed, co-overexpression of Grb7 with EGFR or Her2 is related to cancer progression and malignancy, therefore, Grb7 serves a prognostic marker and therapeutic target in cancers. However, it remains largely unknown the regulatory and signaling mechanisms of Grb7 in regulating these various cellular functions. Here, we observed a novel interaction between Pin1 and Grb7 by a yeast two-hybrid screening. The prolyl cis/trans isomerase Pin1 functions as a regulator to isomerize the pSer/Thr-Pro bond(s), which conferring a conformational change and leading to alterations in protein functionality, stability, and/or intracellular localization. By mutational analyses, we found that Pin1 enables directly interacting with Grb7 through the phospho-Ser194-Pro motif on Grb7 and the WW domain of Pin1. In addition, knockdown of Pin1 expression through lentiviral-mediated gene silencing could prolong Grb7 protein stability. Utilizing cycloheximide pulse-assay and quantitative RT-PCR analysis, we conclude that Pin1 could negatively regulate Grb7 protein stability at the post-translational level. In fact, we found that Pin1 could exert its isomerase activity to modulate Grb7 protein expression in an ubiquitin/proteasome-dependent proteolysis pathway. Collectively, our data suggested that the Pin1-Grb7 complex formation enables negatively regulating tumor progression and/or malignant processes due to the influence of Grb7 protein expression.

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P247

Board Number: B378

NR2F1 regulates epithelial identity and maintains quiescence of a stem cell-like subpopulation of pre-malignant mammary cells.

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Invasive tumor cells are known to be able to complete all steps of the metastatic cascade. However, new evidence shows that pre-malignant cells (from lesions histopathologically defined as non-invasive, i.e. DCIS, ADH) can disseminate and, as disseminated tumor cells (DTCs), enter a non-proliferative state for prolonged periods. Early DTCs are proposed to contribute to metastasis, but the mechanisms that would allow cells considered sessile to complete all steps of metastasis are unknown. Here we report that the orphan nuclear receptor NR2F1 is downregulated in human pre-malignant and malignant lesions. Further, in MMTV-ErbB2+ pre-malignant mammary epithelial cells (MECs) downregulation of NR2F1 activates a motile phenotype and this is coincident with the detection of circulating tumor cells (CTCs) and DTCs in lungs and bone marrow. Knock down of basal NR2F1 levels in pre-malignant ErbB2+ MECs induced cell motility, loss of laminin-V deposition, β -catenin delocalization from the membrane and dramatic loss of E-cadherin junctions. Interestingly, TWIST, SNAIL and ROR1 mRNA levels became upregulated upon NR2F1 depletion. These results suggest that NR2F1 expression maintains epithelial identity and suppresses epithelial-mesenchymal transition (EMT), possibly by blocking WNT signaling. Knock down of NR2F1 in ErbB2+ pre-malignant MECs also enhanced mammosphere formation efficiency and this was accompanied by upregulation of the pluripotency transcription factor NANOG. These findings suggest that NR2F1 limits the spreading of ErbB2+ pre-malignant cells and it promotes a differentiated epithelial identity by promoting quiescence and limiting the activation of a stem cell-like program. Our findings provide for the first time evidence that NR2F1 functions in mammary epithelial cells as a suppressor of pluripotency and dissemination during early stages of tumor progression. We propose that therapies that might restore expression of NR2F1 might allow limiting early dissemination and the progression to metastasis of already disseminated tumor cells.

P248**Board Number: B379****Bundling of the actin cortex by Erk phosphorylated Eps8 is essential for high cortex tension and the bleb-based migration of melanoma cells.**J. Logue¹, A. Cartagena-Rivera², R. Chadwick², C. Waterman³;¹NHLBI/NIDCD, National Institutes of Health, Bethesda, MD, ²NIDCD, National Institutes of Health, Bethesda, MD, ³National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD

Melanoma is a cancer that is known for its rapid spreading and low survivability. Within the confines of tissues, a growing body of evidence suggests that cancer cells frequently use blebs to migrate.

Melanoma cells are hyper-contractile because of an activating mutation in the Erk signaling pathway (B-Raf V600E) that pre-disposes cells to blebbing. Epidermal growth factor pathway substrate 8 (Eps8) is an actin bundling and capping protein whose capping activity is inhibited by Erk phosphorylation. We hypothesized that Eps8 may act as a key effector of Erk to modulate actin cortex mechanics and thereby mediate bleb-based migration of cancer cells. We used live cell imaging of fluorescent probes and atomic force microscopy (AFM) to test this hypothesis in human melanoma A375 cells. We found that Eps8 rapidly localized to newly formed bleb membranes, co-incident with the assembly of cortical F-actin, suggesting it regulates actin in blebs. When cells were confined under agarose, they migrated rapidly in the direction of a very large bleb (50% or more of total cell area), which we call a "leader bleb," and which exhibited co-localization of Eps8 with actin along its cortex. Depletion of Eps8 by siRNA reduced leader bleb size and prohibited bleb-based migration. Re-expression of wild-type Eps8 but not a bundling-defective mutant rescued leader bleb size, indicating a critical role for actin bundling by Eps8 in bleb-based migration. Re-expression of a capping-deficient Eps8 mutant in Eps8 knockdown cells rescued leader bleb size, but re-expression of a mutant with constitutive capping activity induced by substitution of Erk phosphorylation sites (S624A/T628A) did not. This suggests that down-regulation of Eps8 capping activity by Erk is required for leader bleb formation. Total Internal Reflection Fluorescence (TIRF) microscopy showed that WT or capping defective Eps8, but not bundling defective or non-phosphorylatable Eps8 could effectively bundle the actin cortex, suggesting that the capping activity of Eps8 antagonizes its efficiency as an actin bundler. Using a Fluorescence Resonance Energy Transfer (FRET)-based biosensor of Erk activity we found that Erk activity exhibited a gradient from front-to-back in leader blebs. Atomic Force Microscopy (AFM) of blebbing cells showed that cortex tension was reduced in cells over-expressing a dominant negative bundling-defective Eps8 mutant or non-phosphorylatable Eps8, while cells over-expressing a dominant negative capping-deficient Eps8 had elevated cortex tension. Collectively, our results suggest a model in which a gradient of Erk activity in leader blebs locally regulates Eps8 to create a gradient of actin bundling and high cortex tension to drive the bleb-based migration of melanoma cells.

P249**Board Number: B380****Contact inhibition of locomotion in a fibrillar-like microenvironment during breast cancer progression.**D.F. Milano¹, S.K. Muthuswamy², A.R. Asthagiri^{1,3};¹Department of Chemical Engineering, Northeastern University, Boston, MA, ²Princess Margaret Cancer Center, University of Toronto, Toronto, ON, ³Department of Bioengineering, Northeastern University, Boston, MA

Recent work demonstrated that cancer cells invade through a 3-D microenvironment composed of fibers. Tumor-associated fibroblasts help facilitate this invasion by aligning fibers perpendicular to the tumor-stromal interface. Invasion of the tumor boundary is more efficient when metastatic cancer cells migrate along these fibers, which serve as adhesive, directional cues out of the primary tumor. Yet, much of our current understanding about how cancer cells acquire invasive capabilities is based on studies using non-fibrillar, standard 2-D environments. A hallmark of invasive cancer cells is the loss of contact inhibition of locomotion (CIL). To begin to examine the nature of CIL in confined, fibrillar-like microenvironments, we utilized surfaces with micropatterned stripes whose high aspect ratio emulates a protein fiber. Time-lapse microscopy was used to quantitatively compare homotypic interactions between non-transformed mammary epithelial cells (MCF-10A) and metastatic breast cancer cells (MDA-MB-231). Cells were confined on patterned lines with widths from 6 to 33 microns. Pairwise collisions between cells were imaged in real-time and scored as bouncing or sliding collisions using frame-by-frame image analysis. The fraction of sliding collisions was measured as a representative metric to quantify the loss of CIL and reported as a function of patterned line width. Our data reveal that MDA-MB-231 cells do overcome CIL even in confined 1D microenvironments. In fact, our results demonstrate the ability of these metastatic cells to evade CIL is significantly enhanced in more confined 1D-like microenvironments, suggesting the migratory advantage of triple negative breast cancer cells may be particularly well tuned to the spatial constraints of the local tumor microenvironment. Additionally, we examined the role of partitioning defective homolog 3 (PAR3), a known suppressor of metastasis in breast cancer and regulator of CIL *in vivo*, by itself and in combination with the proto-oncogene ErbB2, on CIL in confined microenvironments. Our analysis showed PAR3 knockdown alone had no effect on the observed frequency of sliding collisions but did decrease the duration of bouncing collisions to a level indistinguishable from MDA-MB-231 cells. Activation of ErbB2 alone in MCF-10A cells did lead to a slight increase the ability of cells to slide, however, the greatest increase in sliding ability was observed when ErbB2 was activated in PAR3 knockdown cells. The cooperative nature of ErbB2 activation and PAR3 knockdown to facilitate a more invasive phenotype agrees with previous work performed *in vivo* and validates this platform as a novel tool to quantitatively investigate CIL in a physiological *ex vivo* microenvironment.

P250**Board Number: B381****Role of aberrant vimentin expression in early and late events of human oral cancer.**M. Vaidya¹, C.C. Dmello¹, S.S. Sawant¹, H. Alam²;¹Vaidya Lab, ACTREC-TMC, Navi Mumbai, India, ²Dept. of Molecular and Cellular Oncology, The University of Texas M D Anderson Cancer Center, Houston, TX

Vimentin is an intermediate filament protein predominantly expressed in mesenchymal cells (Lazarides E, 1982). Its association has been shown with progression of many cancers including carcinomas (Satelli A, Li S, 2011). In our earlier study, we had seen the expression of vimentin in oral leukoplakia and submucous fibrosis which are known premalignant lesions for human oral cancer (SS Sawant et al, 2013). Its presence in premalignant lesions suggests the possibility of its role in early oncogenesis. Hence we overexpressed vimentin in human oral premalignant derived cell line to ask if vimentin is one of the causes or just a consequence of the process of neoplastic development. Although no difference was seen between the transformation potential of vimentin overexpressing vs vector control clones using soft agar colony forming assay, critical EMT and stemness related molecular changes were observed amongst them using reverse transcriptase PCR. Therefore further chemical carcinogenic stimuli were given using benzopyrene to both the clones till they showed an anchorage-independent state of growth in soft agar. Remarkably vimentin expressing clones showed more number of soft agar colonies as compared to vector control clones. Vimentin is well reported in late events of cancer progression but the molecular pathway underlying it is unclear hence we downregulated vimentin in oral cancer derived cell line using shRNA technology. We found increased surface levels and decreased biological turnover of $\beta 4$ integrin in absence of vimentin. It was seen that the normal function of $\beta 4$ integrin as mechanical adhesive device was enhanced while its signaling function remained unaltered upon vimentin downregulation. Another important proteins that we found altered upon vimentin knockdown, were the pair of K5/K14. Our data suggests that vimentin may regulate the pair of K5/K14 transcriptionally through delta p63 alpha to contribute in transformation potential of the cancer cell. Also similar correlations in expression levels of vimentin/beta4 and vimentin/K14 were seen in human oral tumor samples. Collectively this study provides insights into role of vimentin in the process of human oral oncogenesis.

P251**Board Number: B382****Identification of an ErbB2+ early disseminating pre-malignant cancer cell sub-population with metastatic potential.**K. Harper¹, M. Sosa¹, H. Hosseini², A. Avivar-Valderas¹, N. Chandandeep¹, R.J. Davis³, C. Klein², D. Entenberg⁴, J.S. Condeelis⁵, E. Farias¹, J.A. Aguirre-Ghiso^{6,7};¹Hematology and Oncology, Icahn School of Medicine at Mount Sinai, New York, NY, ²Experimental Medicine and Therapy Research Faculty, University of Regensburg, Regensburg, Germany, ³Univ Massachusetts Med Sch, Worcester, MA, ⁴Albert Einstein College of Medicine, Bronx, NY, ⁵Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY, ⁶Black Family Stem Cell Institute, Manhattan, NY, ⁷Division of Hematology and Oncology, Mount Sinai School of Medicine, Department of Medicine, NY

Recent clinical evidence suggests that cells are capable of disseminating from “pre-invasive” breast cancer lesions. It has been proposed that these early disseminating cancer cells (eDCCs) lodge into secondary sites, enter a state of dormancy, escape conventional therapy, and potentially evolve through different pathways from the primary tumor to form metastases. However, the mechanisms that endow pre-malignant cells to disseminate and their behavior in target organs remain unknown. Here we show that ErbB2+ pre-malignant cancer cells, while non-tumorigenic are highly efficient in disseminating. While a majority of these eDCCs remain dormant in target organs they are still endowed with a metastatic capacity. We identified an ErbB2^{HIGH}/p38^{LOW}/E-cadherin^{LOW} population of pre-malignant mammary epithelial cells (MECs), which undergo a Wnt-dependent EMT and disseminate. Using intravital imaging of transgenic ErbB2-T-CFP pre-malignant lesions we imaged this process at high resolution revealing that ErbB2^{HIGH}/p38^{LOW}/E-cadherin^{LOW} cells display activate cancer cell motility, invadopodia formation and intravasation capacity. This correlated with CK8/18+/HER2+ circulating cancer cells as well as eDCCs in the lung and bone marrow of mice, a process that was enhanced following 2 weeks of systemic p38 α / β inhibition. We conclude that at pre-malignant stages p38 α / β antagonizes ErbB2 signaling restricting early dissemination of cells, and that early dissemination is a source of predominantly dormant eDCC with metastasis initiating capacity that might contribute to relapse.

P252**Board Number: B383****Heterogeneity in cell-matrix adhesion as an indicator of metastatic state.**A. Fuhrmann¹, T. Tlsty², A.J. Engler¹;¹Bioengineering, University of California, San Diego, La Jolla, CA, ²University of California, San Francisco, San Francisco, CA

Cancer cells have great genetic diversity, which may be reflected in varied metastatic potential. As it has been difficult to determine a comprehensive set of genetic markers to define a metastasizing cell population, we propose a common behavior, i.e. cell-matrix adhesion, that may be differentially

regulated in metastasizing versus non-metastasizing cancer cells. To assess cell-matrix adhesion strength in a heterogeneous population of breast cancer cells, we employed a spinning disc device where cells adhering to matrix-coated substrates were exposed to radially-dependent shear. Within a cell population, cells at the center or edge experience low or high matrix detachment forces, respectively. Highly metastatic cancer cell lines, e.g. MDA-MB-231 and MDA-MB-468, exhibit the same detachment behavior as non-malignant MCF10A and non-metastatic MCF7 cells in the presence of tumor-like concentrations of magnesium and calcium, which regulate integrin activation. However at stromal concentrations of these cations, only the adhesion of metastatic cells was drastically lowered and broadly distributed, suggesting that the most metastatic cells may be more sensitive to lower cation concentrations. Average attachment strength—defined as the shear required to detach 50% of cells, i.e. τ_{50} —and restoration of attachment-homogeneity gradually increased with cation concentration with the greatest sensitivity occurring between 0.01 and 0.50 mM, which falls within the physiological range for both stroma and tumor. Moreover, focal adhesion assembly in MDA-MB-231 cells was highly dependent on cations: at stromal concentrations, cells had more labile adhesions compared to large, stable adhesions at tumor-like concentrations. These changes were absent in non-metastatic cells, indicating that variances in attachment strength and adhesion assembly in the absence of cations may be cell state dependent. Indeed after selecting for strongly attaching metastatic cells, cells maintained their strong attachment phenotype for several days, but stochastically return to heterogeneous adhesion strength over several weeks. Migration and invasion of selected MDA-MB-231 cells were markedly impeded versus their non-selected or non-metastatic counterparts, i.e. sensitivity to cation removal indicates higher invasion potential. These results suggest a potential biophysical mechanism to differentially regulate cell metastasis.

P253

Board Number: B384

Mismatch in cell mechanical properties in co-cultures can lead to enhanced cell motility: A Brownian Dynamics Simulation.

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Recent experiments suggest that the mechanical stiffness of cells and their interaction with their surroundings undergo remarkable changes during tumor progression [1,2]. An intriguing experimental result in this area suggests that the mismatch in the elasticity and adhesive properties between cancer cells and non-cancerous cells in a co-culture may lead to enhanced cancer cell motility [2]. Motivated by this, we study a mathematical model of a 2D co-culture of cells with different stiffnesses and adhesivity using active Brownian Dynamics simulations. We characterize cell motility by studying particle trajectories, mean square displacements and correlation functions. We find that in a binary system of softer and stiffer particles, the softer particles are more motile, and the difference in motility between the two types of particles is enhanced with increase in difference between their stiffnesses. We also find that introducing particle-particle adhesion to the stiffer particles can further increase the motility of the

softer particles. Our study may provide insights into the interplay of mechanical and statistical mechanical properties underlying the enhanced motility of cancer cells during metastasis [2].

References: [1] S. Suresh, Biomechanics and biophysics of cancer cells, *Acta Biomaterialia* 3, 413 (2007).
[2] M. H. Lee, P. H. Wu, J. R. Staunton, R. Ros, G. D. Longmore, and D. Wirtz, Mismatch in Mechanical and Adhesive Properties Induces Pulsating Cancer Cell Migration in Epithelial Monolayer 102, 2731 (2012).

P254

Board Number: B385

Joined forces of cancer cells and fibroblasts against the basement membrane.

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In carcinoma in situ, the basement membrane represents a physical barrier that prevents spreading of primary tumor to adjacent tissues. It is believed that cancer cells perforate basement membranes. However, stromal cells such as carcinoma-associated fibroblasts also secrete matrix proteinases. Therefore, the question is who is invading whom – do cancer cells invade stroma or possibly stroma is invading tumor cells? Using human colon cancer cells and primary human fibroblasts isolated from tumors and adjacent normal tissues, we addressed if cancer cells and fibroblasts are invading the basement membrane simultaneously or they work together but have distinct functions. Analyzing human colon cancer samples, we observed that invasive tumors contain higher amount of fibroblasts in general and higher proportion of CAFs (α SMA+) compared to non-invasive tumors or healthy tissues. We isolated fibroblasts from fresh human colon tumors of different stages (CAFs) and from the adjacent normal tissues (NAFs). A combination of markers was used to discriminate fibroblasts from other cell types to validate the purity of isolated cells and to discriminate NAFs from CAFs. In co-culture experiments on Matrigel-coated transfilters, both NAFs and CAFs induced migration and invasion of HT29, intrinsically non-invasive colon cancer cells. On contrary, in an assay containing native, mesenteric basement membrane that separates cancer cells on one side and fibroblasts embedded in collagen I on the other, we found that only CAFs are able to stimulate invasion of cancer cells. CAFs stimulated invasion of cancer cells when physically present in the assay and, in a lesser amount, via paracrine ways. Proteomic study using SILAC, showed that CAFs secrete more proteases, extracellular matrix proteins, and proteins that modify the matrix compared to NAFs, pointing to a matrix-remodeling role in invasion. Live cell imaging showed that fibroblasts and cancer cells are communicating through the basement membrane via cellular protrusions long time before the actual translocation of cancer cells is detected. We are currently testing a role of CAF-derived molecules in basement membrane remodeling in order to dissect the interplay between cancer cells and CAFs in basement membrane invasion.

P255**Board Number: B386****Cortactin, a key regulator of tumor progression, promotes exosome secretion.**

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Exosome secretion from cancer cells is thought to drive cancer aggressiveness. Presumably, exosome secretion is a regulated event; however little is known about the molecular regulators or how this is altered in cancer. In this study, we show that the tumor-overexpressed cytoskeletal protein cortactin plays an important role in controlling exosome secretion. Inhibition or overexpression of cortactin respectively inhibited or upregulated the number of exosomes secreted from cells. Proteomic analysis indicated that cortactin only affected the number of vesicles but not their cargo content. Live imaging experiments revealed that cortactin controls exosome secretion by controlling trafficking from late endosomes. These data may explain the reported association of cortactin overexpression with tumor aggressiveness and poor patient prognosis.

P256**Board Number: B387****Effect of Vitamin C on the Cytotoxicity of Vemurafenib or Doxorubicin Human Malignant Melanoma Cells.**

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Objective: The goal of this study is to identify the effect of vitamin C on the cytotoxicity induced by Vemurafenib or Doxorubicin in human malignant melanoma cells. **Methods:** The effect of vitamin C on the cytotoxicity induced by Vemurafenib or Doxorubicin were examined by cell viability assay, wounding healing assays, and Transwell migration assay to measure its effects on cell proliferation, migration, and invasion. The apoptotic cell percentage were determined by flow cytometry. **Results:** Vitamin C alleviated the cytotoxic effects of Doxorubicin or Vemurafenib on proliferation, migration, and invasion of human malignant melanoma cells when the concentration of vitamin C was relatively low (=5mM). Vitamin C significantly reduced the percentage of apoptotic cells induced by Doxorubin or Vemurafenib when the concentration was low (1mM). **Conclusion:** Vitamin C showed the dual roles in the cytotoxicity induced by Vemurafenib or Doxorubicin in malignant melanoma cells depending upon the concentrations. Vitamin C alleviated the cytotoxic effect of Doxorubicin or Vemurafenib on human melanoma cells when the concentration was low. However, when the concentration of vitamin C become higher, it enhanced the cytotoxic effect of Doxorubicin or Vemurafenib on malignant melanoma cells.

P257**Board Number: B388****Rosehip (*Rosa canina*) extracts prevent serum-stimulated migration of human glioblastoma cells.**F.S. Ogunsemowo¹;¹Department of Biology, North Carolina AT State University, Greensboro, NC

Glioblastoma multiforme (GBM) are the most common and most aggressive intracranial tumors. Malignant gliomas are characterized by their diffuse invasion of surrounding brain tissue. Despite modern diagnostics and treatments the median survival time is less than a year; and over the past decades no significant increase in survival of patients suffering from this disease has been achieved. Thus, alternative treatment and prevention options are necessary to combat this disease. Natural products represent a source of potential chemical molecules exhibiting anti-oncogenic properties. An example is Rosehip (*Rosa canina*); rosehips are blossoms from the wild rose and frequently used as an herbal medicine. Recently our laboratory has shown that rosehip extracts exert anticancer activities *in vitro*, by inhibiting proliferation of GBM cells. Preliminary data from our laboratory shows that rosehip extracts are able to prevent breast cancer cell migration. Therefore, we investigated the efficacy of rosehip extracts in preventing cell migration of human GBM cell lines. We hypothesize that GBM cell migration will be prevented when exposed to rosehip as a result of interference of cell signaling pathways. Wound healing assays were performed to examine the anti-migratory effects of rosehip extracts (1mg/ml) on human GBM cell lines, U-251 MG and U-87 MG. The data demonstrate that rosehip extracts facilitate inhibition of serum-stimulated cell migration more effectively than MAPK (UO126) and AKT (LY294002) inhibitors. We extended this investigation by examining whether rosehip extracts could prevent EGF-induced cell migration. Rosehip extracts did not prevent EGF-induced migration, suggesting that an alternative mechanism is promoting EGF-associated migration in these cells. These data suggest that rosehip extracts may serve as an alternative therapy for treating certain populations of malignant gliomas.

P258**Board Number: B389****Metastasis Upregulated Genes Have Distinct Function in *C. elegans* Cell Migrations.**S.A. Vetrone^{1,2}, M. Kato², P. Sternberg²;¹Biology, Whittier College, Whittier, CA, ²HHMI, Biology and Biological Engineering, California Institute of Technology, Pasadena, CA

Cell migration is vital for normal animal development but also contributes to the invasive spreading of early stage metastatic cancer. From two published databases, we compiled 107 genes unregulated in either breast cancer or melanoma metastases and investigated their requirement in two *Caenorhabditis*

elegans cell migrations: the male linker cell (LC) and hermaphrodite distal tip cells (DTC) which have similar functions as gonadal leader cells that undergo a complex migration while pulling non-motile followers. We performed an RNAi screen to identify genes implicated in normal LC and DTC migrations. Thirty-two genes from the metastasis list were required for the cell migration of which 13 genes effected the migration of both LC and DTC, 18 genes effected only LC migration, and 4 genes only DTC migration. The genes used by both cell types corresponded to genes involved in cell cycle activity, adhesion, cytoskeleton organization, protein degradation activity, spliceosome activity, ubiquitin-like modification and function, and peptidase inhibition. The genes used by only the LC corresponded to genes involved in adhesion, cytoskeletal organization, methyltransferase activity, metalloproteinase activity, and signaling. Those genes used by only DTC corresponded to genes involved in signaling, peptidase inhibition, and utrophin activity. The significant differences among the developmental cell migrations and the overlap in genes shared between the two metastases underscores the value of characterizing diverse genes and considering cell type in developing treatments.

P259

Board Number: B390

DIBUTYLTIN-INDUCED ALTERATIONS OF INTERLEUKIN 1 beta SECRETION FROM HUMAN IMMUNE CELLS.

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Dibutyltin (DBT) is an organotin compound that is used as a stabilizer in polyvinyl chloride (PVC) plastics (including pipes that distribute drinking water and bottles) and as a de-worming agent in poultry. DBT is found in human blood samples, and DBT exposures alter the secretion of tumor necrosis factor alpha (TNF α) from lymphocytes. Interleukin 1 beta (IL-1 β) is a pro-inflammatory cytokine that promotes cell growth, tissue repair, and immune response regulation. Produced predominately by both monocytes and macrophages, IL-1 β appears to increase the invasiveness of certain tumors. The aim of the current study is to determine whether exposure to DBT alters the secretion of IL-1 β from increasingly reconstituted preparations of human immune cells. We examined whether exposure to DBT concentrations of 0.05 to 5 μ M after 24h, 48h, or 6 days alters IL-1 β secretion in a preparation of highly enriched human NK cells, a monocyte-depleted preparation of human peripheral blood mononuclear cells (PBMCs) (MD-PBMCs), PBMCs, granulocytes, and a preparation combining both PBMCs and granulocytes. The levels of IL-1 β were monitored using an enzyme-linked immunosorbent assay (ELISA). The results indicated that DBT alters IL-1 β secretion from all of the above cells preparations. The high concentrations of DBT (5 and 2.5 μ M) decreased the secretion of IL-1 β , while some of the lower concentrations of DBT (0.1 and 0.05 μ M) increased the secretion of IL-1 β . The concentrations and lengths of exposure to DBT that caused statistically significant increases in IL-1 β secretion from human immune cells varied from one donor to the next. Therefore, the data indicates that DBT-induced alterations of IL-1 β secretion from immune cells may potentially affect immune function and cancer

invasiveness. An additional aim is to examine the signaling pathways involved in the DBT-induced increases in IL-1 β secretion from MD-PBMCs, which are a preparation that primarily consists of T and NK lymphocytes. MD-PBMCs will be treated with inhibitors of pathways that regulate IL-1 β secretion (IL-1 β cleavage inhibitor, ERK $\frac{1}{2}$ pathway inhibitors (PD98059, U), p38 inhibitor (SB202190), and NF κ B inhibitor (BAY 11-7085)) 1h prior to adding DBT concentrations of 0.1 and 0.05 μ M for 24h.

P260

Board Number: B391

Oncogene-expressing cells collaborate to evade microenvironmental restraint.

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Healthy tissue can harbor a high frequency of “occult” cancer; in which isolated transformed cells do not progress into overt tumors. We hypothesized that multiple transformed cells can collaborate to accelerate the earliest stages of tumor progression.

To test this hypothesis, we studied the growth trajectories of small (~8 cell) 3D microtissues comprising wild-type MCF10A (WT) mammary epithelial cells intermixed with MCF10AT (Ras) cells that expressed low levels of a constitutively active H-Ras oncogene. H-Ras expression confers cells with increased rates of cell motility and proliferation. During culture in Matrigel over 7 days, we quantified the growth rates of each cell type by tracking fluorescent nuclear reporters of cell identity by confocal microscopy.

Mixed tissues containing single Ras cells showed a broad diversity of growth trajectories over 7 days of culture. Ras cells either expanded rapidly through the WT population (an “escape” trajectory), did not expand through the population (“restraint”), or reached a steady-state fraction of the cell population while also triggering rapid expansion of both the WT and Ras cell populations (“collaboration”). In contrast, cell assemblies containing two or more Ras cells were far more likely to show collaboration rather than restraint trajectories.

Our data suggests that a tipping point can exist wherein an increase in the frequency of oncogene-expressing cells amongst wild-type neighbors triggers a transition between restrained and collaborative proliferation of entire cell collectives.

P261**Board Number: B392****Mechanisms of Transendothelial Migration by Invasive Breast Carcinoma Cells from Patients.**J. Pignatelli¹, S. Goswami^{1,2}, J.G. Jones^{1,3,4}, X. Chen¹, J.S. Condeelis¹, M.H. Oktay^{1,4};¹Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY, ²Department of Biology, Yeshiva University, New York, NY, ³Department of Epidemiology and Population Health, Albert Einstein College of Medicine, Bronx, NY, ⁴Department of Pathology, Albert Einstein College of Medicine, Bronx, NY

The majority of breast cancer related deaths are not due to the primary tumor, but rather to the spread of distant metastatic tumors. Our lab has previously described the identification of the tumor microenvironment of metastasis (TMEM) in mouse and human mammary tumors, sites where transendothelial migration (TEM) occurs and therefore intravasation. The constituent cells of TMEM are an endothelial cell, a perivascular macrophage and an invasive Mena-over expressing tumor cell in direct contact. TMEM are present in human invasive breast tumors and the density of TMEM is positively associated with the risk of developing metastases.

Using invasive ductal carcinoma cells of the breast obtained from patients by fine needle aspiration, we demonstrated that intravasation-directed transendothelial migration of these cancer cells requires macrophages and, depending on clinical subtype, involves either paracrine, or both paracrine and autocrine signaling. Compared to the total population of primary breast cancer cells assayed, cells capable of transendothelial migration expressed relatively high Mena^{INV} and low Mena^{11a} levels, independently of clinical subtype. Mena^{INV} and Mena^{11a} are functionally distinct isoforms of Mena, a key regulator of motility and invasion. Furthermore, relative Mena^{INV} expression correlated with the density of TMEM, which were previously shown to correlate with risk of metastasis in patients. Our data reveal targetable signaling events required for transendothelial migration of human breast cancer cells, and indicate that relative Mena^{INV} levels and TMEM frequency are correlated prognostic markers of metastasis and therapeutic targets for most human breast cancers.

P262**Board Number: B393****Transendothelial migration of uveal melanoma cells.**M.D. Onken¹, J. Li¹, J.A. Cooper¹;¹Department of Cell Biology and Physiology, Washington University School of Medicine, Saint Louis, MO

Uveal melanoma (UM) is cancer arising from the pigmented layers of the eye that is highly metastatic and only spreads through the bloodstream. Almost half of UM patients develop distant metastatic disease, most often in the liver, even after the tumor-bearing eye is completely removed. The key to understanding and targeting UM metastasis is in understanding how circulating tumors cells enter and

exit the bloodstream and then invade and colonize distant organs. We use primary human dermal microvascular endothelial (HDMVEC) monolayers grown on polyacrylamide soft substrates that mimic the physiological stiffness of normal tissue as a model of the blood vessel wall. To these monolayers, we add UM cells and follow their transendothelial migration. We found that UM cells transmigrate via a unique route that is distinct from the transendothelial migration common to immune cells. During this multistep process, UM cells identify HDMVEC cell-cell junctions and then intercalate between adjacent HDMVECs, taking on a flattened morphology while maintaining contacts with the adjacent endothelial cells. UM cells remain intercalated for an extended period in a state reminiscent of vasculogenic mimicry. After intercalation, UM cells extend invasive projections beneath adjacent HDMVECs, and use these to migrate beneath the monolayer. Immunofluorescence in fixed monolayers revealed cortical actin networks with strong cortactin staining within these invasive projections. We used UM cells expressing F-tractin, a fluorescent fusion protein that specifically binds filamentous actin without disrupting actin function, to image of actin dynamics in live cells. Time-lapse confocal images revealed complex actin-rich projections invading and sampling the interface between the endothelial monolayer and the substrate. Dynamic actin cytoskeleton reorganization was also apparent as cells migrated under the monolayer. We used blocking antibodies to identify cell adhesion molecules that were involved in UM transmigration. We found that blocking VCAM1 inhibited early intercalation but enhanced overall migration, suggesting VCAM1 might be necessary for initiation and maintenance of intercalation. BAP1 is the major metastasis suppressor in human UM tumors. We knocked down BAP1 in our uveal melanoma cell lines, and found that loss of BAP1 had no significant effect on initiation or intercalation of UM cells. However, overall migration was significantly enhanced by BAP1 knockdown. Our studies show that UM transendothelial migration occurs in two phases: vasculogenic intercalation that depends upon VCAM1 adhesion, and subsequent invasion under the monolayer, which is regulated by BAP1.

P263

Board Number: B394

Dissecting the Mechanism of Tumor Cell and Macrophage Streaming Towards Blood Vessels.

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In vivo, breast tumor cells have been observed to migrate with macrophages on collagen fibers towards blood vessels in a process known as “streaming.” Mechanistically, the interactions of the macrophages and tumor cells have been characterized to involve an EGF and CSF-1 paracrine loop with macrophages secreting EGF and tumor cells releasing CSF-1 to attract each other. However, the signaling molecule that is responsible for the directional migration of both the tumor cells and macrophages towards the blood vessel has not been determined.

By plating MTLn3 breast tumor cells and primary bone-marrow derived macrophages (BMMs) on micro-patterned substrates that mimic the diameter and composition of the collagen fibers found in vivo, we

observed that tumor cells and macrophages align together to form “pairs.” However, the tumor cells and macrophages did not demonstrate directional or biased migration towards one direction within the assay. However, directional migration of tumor cells could be established when beads coated with either NIH3T3 fibroblasts or human umbilical vein endothelial cells (HUVEC), both known sources of HGF, were placed at one end of the micro-patterned “collagen fibers” within the assay. This directional migration was inhibited with an HGF inhibitor, suggesting that HGF is the molecule responsible for the directional migration of tumor cells towards blood vessels. This combination of cells; tumor cell, macrophage and endothelial/fibroblast, when placed on a linear substrate, reconstituted streaming in vitro with speed, directional persistence and cell migration characteristics like those observed in vivo during tumor cell streaming toward blood vessels.

P264

Board Number: B395

A Novel Bone Bioreactor Used to Model Bone Metastasis Ex Vivo.

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Bone metastases are incurable. Understanding what drives cancer to metastasize to bone and identifying treatments that eliminate bone metastasis are essential to improving the survival and quality of life of cancer patients with metastasis to bone. The current methods used to study bone metastasis are restricted to in vitro tissue culture models and to in vivo animal models, both of which have several limitations. The in vitro tissue cultures lack the 3-D environment of heterogeneous cell types of the bone and marrow, and in vivo animal models often are limited by the confounding primary tumor burden. Both options generally are not applicable to rapid screening aimed at targeting bone metastases. In this interdisciplinary project, we use a novel bone bioreactor to culture mouse bone explants, study bone metastases, and develop therapies to help breast cancer patients that have developed bone metastases. The objective of this research is to develop an experimental system that preserves the 3-D environment and heterogeneous culture conditions (bone, marrow, and cancer cells) within the physiological context of an intact bone environment and apply the technology to develop faster screening techniques than the ones available in current animal models.

We will use this ex vivo bone culture bioreactor to identify the molecular factors that contribute to develop bone metastases and to aid in the screenings of new drugs aimed at targeting bone metastasis in breast cancer patients. We will validate the bioreactor as a means to understand the stages of metastatic tumor colonization, progression, and response to therapies. After validation in a murine model, our bioreactor will make it possible to study metastatic cancer progression temporally and independently from primary tumor growth. Because this system is amenable for investigating bone colonization by multiple cancer types, this study also has general application beyond breast cancer. Due

the usage of bone explants and vibrational technology that is currently available to patients, this study has high translational value.

Due the limitations of current methodologies and lack of exploration to improve them, our unique interdisciplinary perspective to attack this problem will result in high impact publications in the fields of cancer and bone behavior.

P265

Board Number: B400

Multiclonal Seeding is a Frequent Route to Metastatic Spread.

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Introduction: A foundational concept in metastasis research is that primary tumors first dissociate into single cancer cells in order to seed distant sites. The requirement for a single-cell seed forms an important tenet for common molecular models of metastasis including the epithelial-mesenchymal transition. However, the experimental evidence for the single-cell seed model of metastasis remains limited. Furthermore, circulating tumor cell clusters are detectable in the bloodstream and therefore multiclonal seeding events are also possible. In this study, we experimentally isolated the relative contributions of single and multiclonal seeds and uncovered that multiclonal seeding is a frequent route to metastatic spread.

Methods: We devised a lineage tracing strategy using Rainbow transgenic mice to reveal the clonal origin of lung metastases in MMTV-PyMT mice, a commonly used mouse model of metastatic breast cancer. Primary PyMT tumor organoids that express a genetically encoded Rainbow construct were treated with Cre in order to induce random assortment of color labeling, and then injected orthotopically into the mammary fat pad of host mice. Lung metastases were then scored for the number of unique colors.

Results: If lung metastases arise exclusively from single seeds then each lung metastases should express only one color. Surprisingly, we observed frequent multicolor lung metastases (mean 32%, maximum 61%, N=998 metastases). By implanting tumor organoids labeled with distinct colors into separate flanks of host mice, we observed that these multiclonal seeding events occur predominantly in a single step rather than by serial colonization of single cells. To address why multiclonal seeding might be advantageous, we flow sorted trypsinized tumor cells, gating by size. This enabled us to obtain fractions of single cells and cell clusters of increasing size distributions. Interestingly, we observed that cell clusters had 10-fold higher colony formation and superior survival relative to isolated single cells. Work is underway to test the lung colonization potential of single cells versus cell clusters using tail-vein metastasis assays.

Conclusions: Our results challenge the requirement for single cell seeding in metastatic colonization and establish the importance of a parallel route for metastatic spread involving collective multiclonal dissemination. Furthermore, our data suggest a fundamental mechanism for cell growth and survival mediated by cell-cell contacts. We suggest that disrupting cell contacts may limit cancer cell survival and metastatic spread.

P266

Board Number: B401

Establishing 3-dimensional Spheroids from the Nasopharyngeal Carcinoma (NPC) cell line HK1 to investigate their Growth and Invasion behavior and test their Sensitivity to Flavopiridol.

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Most anti-NPC drug activity studies have come from work performed with two-dimensional (2D) culture assays. 2D culture systems ignore the fact that NPC cells do not live in isolation but are oriented in a 3D space, establishing contact with the stroma, i.e. extracellular matrix and other cell types such as endothelial cells, fibroblasts and immune cells. Three-dimensional (3D) cultures represent a good compromise between the lack of a microenvironment encountered under 2D culture conditions and the great complexity of the *in vivo* animal models. 3D culture mirrors the tumour architecture and microenvironment more closely than 2D, recreates the oxygen/nutrient gradient with a hypoxic zone and a central necrosis and allows interaction between NPC cells and their stroma, when provided. This close resemblance to the situation encountered *in vivo* facilitates more realistic study of NPC growth, invasion and drug response. The objective of this study is to establish a 3D model from the HK1 NPC cell line to determine the growth and invasion behavior of the spheroids on the collagen matrix and to test the sensitivity of the spheroids to chemotherapeutic drugs. Approximately 5000 NPC cells were seeded onto concave agar and left to form spheroids for 72 hours. Once the spheroids have formed, they were harvested and embedded onto the collagen matrix. Spheroid growth and invasion were documented by taking phase contrast images every 24 hours using an inverted fluorescence microscope. Spheroid growth and invasion were quantified by image analysis software's. The HK1 cells formed compact spheroids within 72 hours. Our observation from the 10 days experiments revealed that spheroids gradually grew and invaded into the collagen matrix, showing that the HK1 spheroids are capable of growth and invasion. We also observed formation of holes in the spheroids, which could be explained by cell death under hypoxia. Progressing from these experiments, the HK1 spheroids were employed to perform a drug sensitivity assay using the chemotherapeutic drug, Flavopiridol. The drug had a dose-dependent inhibition of spheroid growth and invasion. Our future endeavor will be to employ the spheroids to study whether Flavopiridol will sensitize the NPC cells to small molecule inhibitors which targets the intrinsic apoptosis pathway for NPC therapy and to establish spheroids from the Epstein -

Barr virus (EBV) positive NPC cell line, C666-1 to observe if the spheroids exhibit the same growth and invasion behavior as the HK1 or whether the EBV infection dictates for any changes.

P267

Board Number: B402

A novel 3D cell culture system using FP001 for in vitro evaluation of anticancer compounds.

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Recently, a number of approaches have been developed to generate 3-dimensional (3D) cell culture models to mimic in vivo environments for cancer studies; e.g. scaffolds, microcarriers, and spheroids. However, these approaches remain some problems such as applying them into high throughput screening (HTS) systems and improving the efficiency of anti-cancer drug discovery. In this study, we identified FP001 as the polymer materials, and developed a novel 3D cell culture medium using FP001 which has the ability to form cancer cell spheroids in normally-distributed and appropriate size. 3D cell cultures system using ultra-low attachment multi-well plates in combination with FP001 exhibited >3-fold increase in the number of A549 cells after 5-day culture as compared to that without FP001. The positive effect of FP001 was applicable to a wide variety of cancer cell lines and was clearly beneficial for HTS. As for the cell proliferation of HeLa and A549, the 3D culture system was more sensitive to AKT inhibitors and MEK inhibitors, compared with that employs 2D monolayer culture condition. Furthermore, Heparin-binding EGF-like growth factor, an EGF receptor ligand, clearly stimulated the growth of SKOV3 and A431 cells in the 3D culture system, but not in monolayer culture one. In conclusion, we established a novel method for the 3D culture of cancer cells under low attachment condition by using FP001, which was available for HTS and showed high sensitivity to molecularly-targeted drugs, EGF signal inhibitors. Our approach would facilitate the development of novel models for in vitro evaluation of anticancer compounds.

P268

Board Number: B403

Utilizing 3-D Collagen Cultures to Represent In Vivo Cancerous Tumors and to Test Targeted Molecular Imaging Agents (TMIs).

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The goal of this research is to develop in vitro assay methods for the evaluation of targeting molecular imaging agents (TMIs) using confocal fluorescent microscopy (CFM). Culturing cells in two dimensions on a plastic surface results in artificial two-dimensional growth patterns of sheets of cells which do not provide a good model of a tumor growing in vivo. Most cells in the human body interact with a three-

dimensional environment, composed of other cells, matrices, fibrous layers, and adhesion proteins. In order to create a more accurate TMIA screening environment that closely mimics how tumor cells grow in the body, 3-D cultures were utilized. Collagen is a very widely used matrix for 3-D cultures due to its chemical and physical simplicity and thus was used for initial experiments. A549 cells were suspended in a liquid collagen matrix which was allowed to solidify on a glass bottom dish. The cells were then stained with a Targeted Molecular Imaging Agent (TMIA) Cy5.5-RGDyk conjugate and other stains such as NucBlue, MitoTracker Red, and Tubulin Tracker. Visualization of cultures was done using confocal microscopy. The cells grown in collagen culture formed spheroid-like structures which more closely resembled a tumor growing in vivo. These cultures are a better in vitro tumor model than cells growing on a 2D surface. Observation of the cells stained with the TMIA agent showed that the TMIA penetrated well into the spheroid structure and that the TMIA was taken into the cells by an endocytosis-like mechanism. The use of 3D collagen culture should allow more efficient TMIA to be identified and hopefully will lead to identification of TMIA agents that will penetrate and stain tumor tissue in the body.

P269

Board Number: B404

Study on the mechanisms of pipoxolan inhibits the migration and invasion in CL1-5 human lung adenocarcinoma cells by reducing MMP-9 and MMP-2 expression.

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Pipoxolan has been shown to have antitumor effect. However, the effects of pipoxolan on lung cancer cell metastasis remains undecided. This study was to investigate how pipoxolan affect the lung cancer cell migration and invasion. This study investigated the antimigration and antiinvasion effects of pipoxolan on lung adenocarcinoma cancer cells (CL1-5 cells) and its underlying molecular mechanisms. The data demonstrates that pipoxolan does not effectively inhibit the viability of CL1-5 cells. Pipoxolan markedly suppress cell migration of CL1-5 cells by the assessment of wound scratch assay and transwell assays. Treatment of pipoxolan brought about inhibition of migration and invasion of CL1-5 cancer cells by transwell assay. And, these observations were associated with a reduction in the activities and levels of matrix metalloproteinase (MMP)-2 and MMP-9 in CL1-5 lung cancer cells. Lastly, pipoxolan at 5 µg/mL and 10 µg/mL inhibited phosphorylation c-Jun N-terminal kinase (p-JNK) of CL1-5 cells. Moreover, pipoxolan at 10 µg/mL inhibited phosphorylation p38 MAP Kinase (MAPK) of CL1-5 cells. In addition, our results also demonstrate that administration with JNK1/2 inhibitor (SP600125), and p38 inhibitor (SB203580) reduce MMP-2 and MMP-9 expressions in CL1-5 lung adenocarcinoma cancer cells. Based on these results, our conclusions show that treatment of CL1-5 lung cancer cells with pipoxolan down-regulates MMP-2 and MMP-9, which are the down-stream targets of JNK and p38 and play a critical role in CL1-5 cells metastasis. These results show a new therapeutic potential for pipoxolan in anti-metastatic therapy.

P270**Board Number: B405****Podoplanin-expressing CAFs Lead and Enhance the Local Invasion of Cancer Cells in Lung Adenocarcinoma.**S. Neri¹, G. Ishii¹, A. Ochiai¹;¹Pathology Division, National Cancer Center Hospital East, Kashiwa, Japan

Cancer-associated fibroblasts (CAFs) communicate with cancer cells and play important roles in cancer invasion. We previously observed that lung adenocarcinoma patients with podoplanin (PDPN)-expressing CAFs frequently exhibited the local invasion of cancer cells. In this study, we established a novel collagen invasion assay model in which cancer cells and CAFs were co-cultured; we then analyzed the mechanisms governing how cancer cell invasion was promoted by PDPN(+) CAFs. We found that PDPN(+) CAFs invaded the collagen matrix to a greater extent, with more cancer cells invading within tracks created by the CAFs, compared with control CAFs. After intravenous injection in the mouse tail vein, PDPN(+) CAFs invaded and promoted cancer cell invasion into the lung parenchyma, compared with control CAFs. Among the lung adenocarcinoma patients, we observed some cases with PDPN(+) CAFs at the invasive front of the tumor. These cases predominantly exhibited pleural invasion of cancer cells, known as pathological invasiveness. Our results indicated that PDPN(+) CAFs were tumor-promoting CAFs that lead and enhance the local invasion of cancer cells.

P271**Board Number: B406****Plant Polyphenols Reduce Migration Ability of Human Melanoma Cells.**C. Jones¹, C.A. Joseph¹, V.S. Parmar², A.L. DePass¹;¹Department of Biology, Long Island University, Brooklyn, NY, ²Department of Chemistry, University of Delhi, New Delhi, India

Melanoma is the most serious type of malignant skin cancer due to its ability to metastasize, and appears highly resistant to many forms of cancer treatments. Plant polyphenols have a number of beneficial health effects, and are well noted for their anti-cancer properties. To develop more effective treatments for melanoma, we assessed the chemotherapeutic potential of various plant polyphenol analogs on the migration ability of A375 human melanoma cells as an *in vitro* model. Treatment of A375 cells with polyphenols MLN-1249, MLN-1337, and MLN-2287 resulted in inhibition of cell migration. Employing cell viability assays, the compounds resulted in dose-dependent cytotoxicity at concentrations ranging from 0 to 100 μ M. Using cell invasion assays, we found that the polyphenols inhibited activity on cell migration at concentrations of 1 μ M over a 72 h time period. The inhibition of cell migration correlates to the expression of genes involved in the apoptosis, NF κ B, and epithelial-to-mesenchymal transition pathways. Together, these results show that the polyphenols have the ability to inhibit cell migration, a crucial step of metastasis.

P272**Board Number: B407****An Examination of Obesity-induced Epithelial to Mesenchymal Transition in Melanoma Cells.**S.S. Papas¹, E. Brandon²;¹Biology, Mississippi College, Clinton, MS, ²Biology, Mississippi College, Clinton, MS

Recent studies have shown that obesity worsens the prognosis of several cancers, including melanoma. Evidence suggests that cytokines such as leptin and resistin, promote the growth, epithelial-to-mesenchymal transition (EMT), and subsequent metastasis of melanoma tumor cells. Our research focuses on extending this understanding by discerning the connection between adipocyte's presence and tumor cell proliferation and migration. Additionally, concentrating on the mTOR signaling pathway as a means for regulation of EMT, we seek to add detail to the otherwise vague mechanisms concerning obesity-associated metastasis of solid tumor cells. To test the hypothesis that tumor cell proliferation is increased by the presence of adipocytes, we co-cultured mouse adipocytes and melanoma cells. Control and experimental melanoma cells were counted at three time points during their treatments; comparisons of the tumor cell growth at 0, 24, and 48 hours, revealed enhanced growth as a result of adipocyte presence. Melanoma cells cultured with adipocytes grew faster than the melanoma cells cultured alone. Growth rates were significantly increased in the co-cultured melanoma cells ($13,163 \pm 3.22$ vs. $10,307 \pm 1.17$ cells/mL/day, Student's T-test $p = 0.01$). Additionally, preliminary data suggests that the co-cultured melanoma cells migrate more than melanoma cells cultured alone. Cells going through EMT have increased levels of vimentin and phosphorylated mTOR. Western blots will be used to determine whether the co-cultured melanoma cells follow this pattern. Adipocytes directly promote the growth of melanoma cells and migration also appears to increase as a result of co-culturing. These results support our previous finding of increased melanoma tumor growth in obese mice. This may occur because of mTOR activation, which remains to be determined. Future experiments will examine the expression of the transcription factors SNAIL, Slug, and Twist in co-cultured melanoma cells.

P273**Board Number: B408****Stiffened extracellular matrix and paracrine signaling from stromal fibroblasts synergistically combine to result in both increased tumor cell invasiveness and a change in invasive strategy in a 3-D m.**J.S. McLane¹, L.A. Ligon¹;¹Biology, Rensselaer Polytechnic Inst, Troy, NY

Several changes have been described in the stroma surrounding a tumor, including changes in cellular composition, altered extracellular matrix (ECM) composition and organization, and increases in tissue

stiffness. Tumor cells are influenced by the composition, organization, and mechanical properties of the microenvironment, as well as by signals from stromal cells. The tumor-associated microenvironment can induce changes in tumor cells such as EMT and increased invasiveness. Here we sought to test whether the small increase in stiffness observed in the stroma surrounding a tumor is sufficient to induce tumor cell invasiveness. Further, we sought to determine if signaling from stromal fibroblasts can regulate tumor cell invasion in the stiffened matrix. In order to test the role of physiologically relevant stromal stiffness in initiating tumor invasion, we developed an in vitro model of a tumor in situ in which the stromal stiffness can be controlled independently of collagen concentration. We generated tumor spheroids with a basement membrane-like coating and then encapsulated them in a collagen-I based 3D hydrogel with or without fibroblasts in the “stroma”. We varied the stiffness of the hydrogel from that of normal breast (~200 Pa) to that of observed breast tumor adjacent stiffness (~800 Pa), a difference of less than 5-fold. Our results show a distinct change in invasive cellular behavior with increased stiffness, and a differential regulation of invasion by fibroblast signaling indicating both physical and paracrine links between the altered stroma and tumor behavior.

P274

Board Number: B409

Mechanical Confinement Triggers Glioma Linear Migration Dependent On Formins.

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Glioblastoma are extremely aggressive brain tumors characterized by their resistance to radio- and chemotherapy and their highly invasive properties. Glioblastoma cells have the remarkable capability to insinuate themselves throughout the brain. Since they migrate in a confined environment, we analyzed their movement on micropatterned lines coated with laminin to mimic the tracks that glioma cells follow when invading the brain. We found that they adopted an efficient linear migration mode upon confinement to lines. During this linear migration, antiparallel streams of cells were observed along the axis of the laminin tracks. Within these streams, individual cells adopted a spindle shape and used a 2 phase saltatory motion that depended on microtubules and contractile actin bundles, and involved paxillin-containing adhesions. Surprisingly, we found that this linear migration mode was dependent on formins but independent of Arp2/3. Finally, we found that in confined conditions glioma cells expressed a specific mDia2 isoform offering a promising target for cancer therapy development.

P275**Board Number: B410****Co-culture with mature adipocytes promotes morphological changes and growth of breast cancer cells.**Y. Lee¹, J. Koo¹;¹Department of Pathology, Severance Hosp, Seoul, Korea

The crosstalk between tumor cells and adjacent stroma plays a significant role in tumor growth and progression. In the breast, the main stroma cell types are fibroblasts and adipocytes. Fibroblasts are widely suggested as stimulating the invasion of breast cancer cells. Although emerging evidences suggest that adipocytes contribute to tumorigenesis in breast cancer, its mechanism remains to be elucidated. In this study, we show that mature adipocytes promote morphological changes and growth of human breast cancer cells. Co-culture of MCF-7 (luminal type) with mature adipocytes exhibited spindle-like structures without other stimuli, compared to MCF-7 cells co-cultivated with preadipocytes. Furthermore, MDA-MB-435S and MDA-MB-231 cells, well-known Triple Negative Breast Cancer (TNBC type), co-cultivated with mature adipocytes induced extensive morphologic change and incomplete EMT. However, co-culture with mature adipocytes has no effect on MDA-MB-453 (Her2 type) and MDA-MB 468 (TNBC type) cells. After co-culture of MDA-MB-231 with mature adipocytes, the cancer cell upregulated mRNA and protein expression of vimentin. Five breast cancer cell lines cultured in the presence of mature adipocytes showed an increase in cell proliferation compared to cancer cell alone culture. These data suggest that mature adipocytes can promote tumor growth and metastasis by causing incomplete EMT via phenotypic change of MCF-7, MDA-MB-435S, and MDA-MB-231 cells.

Cancer Therapy 1**P276****Board Number: B411****Identification of natural products with selective activity against triple-negative breast cancer molecular subtypes.**A.J. Robles¹, L. Du², S. Cai², J.B. King², R.H. Cichewicz², S.L. Mooberry^{1,3,4};¹Department of Pharmacology, The University of Texas Health Science Center at San Antonio, San Antonio, TX, ²Natural Products Discovery Group, Department of Chemistry and Biochemistry, University of Oklahoma, Norman, OK, ³Department of Medicine, The University of Texas Health Science Center at San Antonio, San Antonio, TX, ⁴Cancer Therapy and Research Center, The University of Texas Health Science Center at San Antonio, San Antonio, TX

Triple-negative breast cancers (TNBCs) are tumors lacking estrogen receptor/progesterone receptor expression and amplification of HER2. These tumors respond initially to cytotoxic chemotherapy but have a poor prognosis and drug resistance often develops. TNBCs are heterogeneous cancers without

clear molecular targets for the development of new therapies. Genetic subtyping of 587 TNBC patients by Lehman and Bauer (2011 *J Clin Invest*) identified 6 subtypes of TNBC with distinct genetic alterations, allowing for the first time the ability to search for targeted therapies for TNBC subtypes.

High-content screening was used to evaluate 4,336 fungal and 1,392 plant extracts for selective efficacy against 5 TNBC subtypes. We identified 11 extracts with selective cytotoxic and/or antiproliferative activity against cell lines representing the different TNBC subtypes. Bioassay-guided fractionation of a fungal extract yielded a novel compound, maximiscin, which was found to have selective cytotoxic efficacy against the MDA-MB-468 cell line of the basal-like 1 subtype. Additionally, we isolated deguelin, which had selective activity in the MDA-MB-453 cells of the luminal androgen receptor (LAR) subtype.

Cellular studies were conducted to investigate the molecular mechanisms of action of each compound and to potentially identify new pharmacological targets for TNBC. Initial studies showed that maximiscin caused an accumulation of cells in G1. Protein microarray studies gave a preliminary indication that maximiscin increased levels of phospho-p53, which was consistent with the observed G1 accumulation. Additionally, maximiscin caused a reduction of phospho-S6K and phospho-rS6 levels relative to vehicle-treated cells after 8 h of treatment, suggesting inhibition of the mTOR signaling pathway may be involved in its mechanism of action. A small decrease in cellular levels of phospho-Stat1 and phospho-Stat3 was also observed beginning 8 h after maximiscin treatment. Maximiscin induced apoptosis of MDA-MB-468 cells, but DNA double-strand breaks did not occur prior to the accumulation of cleaved PARP, suggesting that DNA damage is not the primary mechanism of action.

Based on published studies of deguelin and the knowledge that LAR TNBC cells are particularly sensitive to Hsp90 inhibition, we hypothesized that deguelin selectively inhibits growth of MDA-MB-453 cells by inhibiting Hsp90 and modulating androgen receptor (AR) function. Preliminary results suggest deguelin modulates AR levels but does not directly bind Hsp90. Studies focused on further defining the mechanisms of action of each of these compounds are ongoing. These results demonstrate that novel compounds with potential therapeutic value for the treatment of TNBC subtypes can be identified from nature.

P277**Board Number: B412****Rosehip (*Rosa canina*) extracts prevent MAPK and AKT-mediated cell proliferation and migration in African American triple negative breast cancer cells.**P. Cagle¹, T. Coburn^{2,3}, A. Shofoluwe², P.M. Martin²;¹Energy and Environmental Systems, North Carolina AT State University, Greensboro, NC, ²Department of Biology, North Carolina AT State University, Greensboro, NC, ³Energy and Environmental Systems, NC AT State University, Greensboro, NC

Triple Negative Breast Cancer (TNBC) is an aggressive form of breast cancer, characterized by its lack of the human epidermal growth factor receptor-2 (HER-2), the estrogen receptor (ER), and the progesterone receptor (PR). The high prevalence of triple negative tumors is more commonly observed in African American (AA) women. Akt and MAPK have been shown to promote cell proliferation and migration in TNBC and MAPK expression may be an underlying mechanism contributing to the generation of chemo-resistance in triple negative breast cancer. Currently, the existing targeted therapy is of minimal benefit in TNBCs. Furthermore, adverse side effects and the emergence of drug-resistant cancer cells are of great concern. Natural products have received growing interest in recent years as an alternative medicine with potential anti-oncogenic properties. Rosehip extracts have been used as dietary supplements to relieve symptoms associated with gastrointestinal disorders and arthritis, and in our laboratory it has been shown to prevent cell proliferation in glioblastomas. This study investigated the efficacy of rosehip extracts in preventing proliferation and migration of an AA triple negative breast cancer cell line (HCC1806). HCC1806 cells treated with rosehip extracts (1mg/mL -25ng/mL) demonstrated a significant decrease in cell proliferation. The observed decrease in cell proliferation was equal to or better than the decrease of cell proliferation observed when inhibitors of the MAPK (U0126, 10 μ M) or AKT (LY294002, 20 μ M) signaling pathways were utilized. Rosehip extracts also demonstrate anti-migratory potential. Additionally, pretreatment of this cell line with rosehip extracts selectively decreased AKT, MAPK, p70S6K, and S6 phosphorylation suggesting these extracts prevent AA TNBC cell proliferation and migration by blocking both the MAPK and AKT signaling mechanisms. Western blot analysis and apoptosis studies demonstrate that rosehip extracts inhibit cell proliferation without promoting apoptosis. To investigate the potential clinical application of rosehip extracts we examined whether rosehip extracts could enhance the chemotherapeutic properties of Doxorubicin (20 μ M). Rosehip extracts enhanced the anti-proliferative effect of Doxorubicin by promoting apoptosis. These data suggest that rosehip extracts are capable of decreasing cell proliferation and migration in an AA triple negative breast cancer cell line. Moreover, rosehip extracts promote apoptosis and demonstrate a synergistic inhibition of cell proliferation, when given in combination with Doxorubicin. This investigation demonstrates that rosehip extracts may serve as either an alternative or complimentary treatment to current chemotherapeutic regimens for TNBC, especially in AA women.

P278**Board Number: B413****Electrophilic Nitroalkenes Cause Degradation of NFκB RelA in Triple Negative Breast Cancer Cells.**C. Woodcock¹, S.R. Woodcock¹, N. Davidson², B.A. Freeman¹, Y. Huang²;¹Pharmacology and Chemical Biology, Univ Pittsburgh, Pittsburgh, PA, ²University of Pittsburgh Cancer Institute, Pittsburgh, PA

Triple negative breast cancer (TNBC), which lacks estrogen receptor (ER), progesterone receptor (PR) and Her2/Neu, is an aggressive and therapy-resistant metastatic cancer that accounts for up to 20% of breast cancer incidence in the US. Women with TNBC are at higher risk for early relapse within 5 years of treatment, and recurrent tumors become more aggressive and invasive. Current chemotherapy for TNBC is limited because of ill-defined targets and poor efficacy; therefore, there is an unmet need for novel therapeutic agents with improved efficacy that can also prevent disease recurrence. Electrophilic fatty acids are endogenously-generated signaling mediators that modulate cell differentiation and proliferation via post-translational modification of functionally-significant nucleophilic amino acids (Cys, His) of transcriptional regulatory proteins such as NFκB, PPAR α , and Keap1/Nrf2. Preliminary studies have revealed that an exemplary electrophilic fatty acid nitroalkene, 10-nitro-oleic acid (OA-NO₂), displays potential therapeutic value in TNBC cells, by preferentially reducing the growth and viability of two TNBC human breast ductal epithelial cell lines (MDA-MB-231, MDA-MB-468), but not normal MCF10A cells. Essential to proliferation and survival, NFκB plays an important role in TNBC development, and its signaling actions are constitutively activated in ER-negative breast cancer cell lines and primary tumors. Notably, the protein level of NFκB RelA/p65 subunit was diminished in TNBC cells upon OA-NO₂ treatment. Therefore, it is hypothesized that electrophilic fatty acids reduce TNBC cell proliferation and survival through down-regulation of NFκB expression and signaling. We have demonstrated that fatty acid nitroalkenes induce caspase-3 activation and down-regulate transcript levels of NFκB-regulated genes, cyclin D1 and the pro-survival genes, bcl-xL and survivin, in TNBC cells. Moreover, nitro-fatty acids promote p65/RelA protein polyubiquitination via nitroalkylation of p65/RelA in TNBC cells. Overall, this study will help to extend our current knowledge of diverse electrophile functions with proteins, and also serve as a prelude to the clinical study of electrophilic nitrated lipids as therapeutic agents that may display a high selectivity for killing TNBC cells/tumors.

P279**Board Number: B414****Differential Sensitivity of Breast Cancer Cell Lines to Diverse Microtubule Targeting Agents.**A.L. Risinger¹, N. Dybdal-Hargreaves¹, S.L. Mooberry^{2,3,4};¹Pharmacology, Univ Texas Hlth Sci Ctr-San Antonio, San Antonio, TX, ²Department of Pharmacology, The University of Texas Health Science Center at San Antonio, San Antonio, TX, ³Department of Medicine, The University of Texas Health Science Center at San Antonio, San Antonio, TX, ⁴Cancer Therapy and Research Center, The University of Texas Health Science Center at San Antonio, San Antonio, TX

Microtubule targeting agents (MTAs) are some of the most important drugs used in the treatment of a wide range of malignancies. New evidence suggests that the anticancer effects of these agents are not only due to antimitotic actions, but also to disruption of interphase signaling processes that require a functional microtubule network. Microtubules play fundamental roles in cellular trafficking, metabolism and cell signaling and it is not unreasonable that in the context of an oncogene driven cancer cell, disruption of microtubule-dependent processes could inhibit essential transport and signaling, ultimately leading to cytotoxicity. If this is the case, then cell lines representing different molecular phenotypes and oncogenic drivers would be expected to be differentially sensitive to diverse MTAs that have subtly different mechanisms of action. To test this, the effects of 6 MTAs, including both microtubule stabilizers and microtubule depolymerizers, were evaluated in 8 cell lines representing the major classes of breast cancer, including triple negative subtypes. An initial observation is that there was no correlation between the cell doubling rate and sensitivity for either antiproliferative or cytotoxic effects of any of the MTAs. This discounts the notion that cell lines are differentially sensitive only by virtue of their proliferation rate. Second, profound differences among the cell lines were seen. Some are intrinsically resistant to all the drugs tested, for example HCC1937 and T47D cells, while most of the other cell lines have a wide range of sensitivities to the different agents. The MDA-MB-231 cell line is sensitive to the antiproliferative effects of each of the MTAs, yet total growth inhibition was achieved with only 2 of the 6 drugs, vinorelbine and eribulin, at concentrations up to 10 μ M. Third, there was no indication of differential sensitivity across this panel to either microtubule stabilizers or destabilizers, but to individual drugs of each of these classes. For example, Hs578T cells are sensitive to eribulin, vinorelbine, paclitaxel and ixabepilone, yet resistant to docetaxel. Fourth, a variety of dose response relationships were observed, including the classic sigmoidal log dose response curves, inverted U-shaped curves, bi and triphasic curves. Together these data show that there are substantial differences among the potency and efficacy of MTAs even at the in vitro level. Our data suggest that in vitro studies can be useful to begin to understand the mechanistic differences among these agents in the context of different oncogenic signatures. These studies can assist in the elucidation of the multiple mechanisms by which disruption of microtubules can impact cell function and ultimately cell viability.

P280**Board Number: B415****Ceramide-induced secretion of exosome-associated breast cancer resistance protein and reduction of doxorubicin resistance in human breast cancer MDA-MB-231 cells.**

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Many breast cancer cells acquire multidrug resistance (MDR) mediated by ABC transporters such as breast cancer resistance protein (BCRP/ABCG2). Here we show that incubation of human breast cancer MDA-MB-231 cells with farnesoid X receptor antagonist guggulsterone (gug) and retinoid X receptor agonist bexarotene (bex) elevated ceramide, a sphingolipid known to induce exosome secretion. The gug+bex combination reduced cellular levels of BCRP to 20% of control cells by inducing its association and secretion with exosomes. Exogenous C6 ceramide also induced secretion of BCRP-associated exosomes, while siRNA-mediated knockdown or GW4869-mediated inhibition of neutral sphingomyelinase 2 (nSMase2), an enzyme generating ceramide, restored cellular BCRP. Immunocytochemistry showed that ceramide elevation and concurrent loss of cellular BCRP was prominent in Aldefluor-labeled breast cancer stem-like cells. These cells no longer excluded the BCRP substrate Hoechst 33342 and showed caspase activation and apoptosis induction. Consistent with reduced BCRP, ABC transporter assays showed that gug+bex increased doxorubicin retention and that the combination of gug+bex with doxorubicin enhanced cell death by more than 5-fold. Taken together, our results suggest a novel mechanism by which ceramide induces BCRP secretion and reduces MDR, which may be useful as adjuvant drug treatment for sensitizing breast cancer cells and cancer stem cells to chemotherapy. Supported by NIH and NSF.

P281**Board Number: B416****The Microtubule Stabilizer Taccalonolide AJ Inhibits EGFR Signaling and Transport in Breast Cancer Cells.**

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Breast cancer is the second most diagnosed cancer in women, with millions of women suffering from the disease worldwide. Microtubule stabilizers are some of the most important drugs used in the

treatment of breast cancer. The taxanes, paclitaxel and docetaxel are some of the most valuable agents used in the treatment of breast cancer. However, there remains a need for new microtubule stabilizers that can overcome some of the limitations of the taxanes. The taccalonolides are a class of microtubule stabilizers isolated from plants of the genus *Tacca*. The taccalonolides bind covalently to microtubules, have the ability to overcome drug resistance due to the overexpression of PgP, and have shown excellent activity in paclitaxel resistant xenograft models. Recent evidence suggests that the ability of microtubule targeting agents (MTAs) to interrupt microtubule-dependent functions in interphase cells contributes to their anticancer actions. The specific signaling events interrupted by MTAs and mechanistic differences among MTAs are only beginning to be investigated. EGFR is overexpressed in 40% of breast cancers and in triple negative breast cancers EGFR overexpression occurs in 75% of cases. Following ligand binding, activated EGFR is internalized and transported to the endosome, events which are dependent on microtubules. The effects of taccalonolide AJ and paclitaxel on microtubule-dependent EGFR trafficking were evaluated in a panel of breast cancer cell lines, including BT549, MDA-MB-468 and T47D. Our results show that taccalonolide AJ and paclitaxel inhibit EGFR internalization causing the receptor to be retained at the cell periphery following ligand stimulation. This impaired trafficking inhibited the phosphorylation of EGFR's downstream targets, as evidenced by lower levels of ERK1/2, AKT and STAT3 phosphorylation. Surprisingly, taccalonolide AJ was much more effective at inhibiting the phosphorylation of these downstream targets. These data suggest that microtubule stabilizers can modulate EGFR pathways in distinct ways. Identification of the specific signaling pathways and downstream targets interrupted by the different MTAs might identify new rational drug combinations. This has the potential to identify patient populations that would benefit from different MTAs based on the specific signaling defects in their tumors and thus better responses. Our study evaluating differences among microtubule stabilizers begins to shed some light into the complex and subtly different mechanisms of action of these drugs.

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Board Number: B417

Reishi sensitizes EGFR-overexpressing inflammatory breast cancer cells to the tyrosine kinase inhibitor erlotinib.

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Inflammatory Breast Cancer (IBC) is the most aggressive, and lethal type of breast cancer. Its lethality stems from its unique ability of forming tumor spheroids, which invade the dermal lymphatics causing the inflammatory phenotype. Within these spheroids, in IBC cell lines and human tissues, the Epidermal Growth Factor Receptor (EGFR) is overexpressed and it is associated with increasing IBC tumor growth rates, invasion and metastasis. Moreover, the high incidence of resistance to EGFR Tyrosine Kinase Inhibitors (TKIs) has greatly diminished the effectiveness of this chemotherapeutic drug. Thus, we aimed to investigate Reishi's therapeutic potential in IBC, focusing on the regulation of the EGFR signaling cascade and its contribution to the IBC cellular response when treated in combination with erlotinib.

SUM-149 IBC cells were treated with various concentrations of erlotinib, Reishi, or their combination for 72h. In order to study the molecular mechanism for the improved effects of the combination treatment we successfully developed an IBC erlotinib resistant cell line, rSUM-149 cells, which were also treated with erlotinib, Reishi or their combination. Treatment effects were tested on IBC cell viability, invasion via Transwell Invasion Assays, cell migration using FluoroBlok™ Cell Culture Inserts, tumor spheroid formation via three-dimensional cell culture, and immunoblots. Statistical analyses include a One-way ANOVA with post-hoc test using the Dunnett's multiple comparisons estimator and p was set at ≤ 0.05 . Our results show that Reishi sensitizes parental and resistant SUM-149 cells to erlotinib treatment after 72h (ANOVA, $F = 104.1$; p

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Board Number: B418

Ganoderma lucidum decreases the phosphorylation of STAT-3 in in vitro and in vivo inflammatory breast cancer models.

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Inflammatory Breast Cancer (IBC) is an aggressive form of breast cancer (BC) with symptoms that include a diffuse redness and swelling of the breast. Unique pathological findings indicate the presence of tumor emboli invading the dermal lymphatics of the breast; a process that is partly responsible for the inflammatory phenotype. However, these tumors produce inflammatory mediators such interleukin-6 (IL-6), which may act as a growth factor to contribute to cancer progression, invasion and metastasis via the activation of the Janus Kinase-2/Signal Transducer and Activator of Transcription 3 (STAT-3) pathway. Our published data demonstrates that the medicinal mushroom, *Ganoderma lucidum* (Reishi), disintegrates tumor emboli and modulates the abundance of proteins involved in invasion cascades. In the current study, we assessed the effects of Reishi on inflammatory markers focusing on the IL-6/JAK-2/STAT-3 pathway both *in vitro* and *in vivo*. SUM-149 IBC and SUM-102 non-IBC BC cells were treated with vehicle, or Reishi at different time points to determine cancer cell viability, invasion via three-dimensional culture assays and protein abundance via immunoblots. *In vivo* studies were carried out in female severe combined immunodeficient (SCID) mice that were treated with vehicle or 7mg/kgBW, 14mg/kgBW and 28mg/kgBW of Reishi for 2wk. SCID mice were then injected with SUM-149 IBC cells in their lower right mammary fat pad and Reishi treatment was continuously administered for 12 wks. Our data from the *in vitro* study suggests that Reishi reduces cancer cell viability, and Reishi has an immunomodulatory role in SUM-149 IBC cell line, demonstrated by its ability to reduce the secretion of IL-6 and reduced phosphorylation of STAT-3 in SUM-149 cells. Cell invasion studies show that Reishi reduces invasion in IBC cells. Interestingly, Reishi reduces SUM-102 BC cell viability; however it does not affect STAT-3 signaling in the cell line. Our *in vivo* results show that Reishi does not change tumor volume, when mice were treated with any concentrations of Reishi. However, the phosphorylation of STAT-3 in tumor lysates significantly decreases in a concentration dependent manner. Future efforts are

focused on understanding the invasion effects on the SUM-102 non-IBC BC cell line, and on the implications of STAT3 inactivation in IBC. This project was sponsored by Title V PPOHA US Department of Education #P031M105050, NIH/NIMHD # 8G12 MD 007583, NIH/NIGMS #P20 GM103475, NIH/NIMHD U54 MD008149.

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Board Number: B419

The Rac Inhibitor EHop-016 Reduces Mammary Tumor Growth, Metastasis, and Angiogenesis.

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The Rho family GTPases Rac and Cdc42 play pivotal roles in actin cytoskeleton reorganization during migration/invasion and thus, metastasis; as well as epithelial to mesenchymal transition, transcription, cell proliferation, cell cycle progression, apoptosis, vesicle dynamics, angiogenesis, and cell adhesions. In many human cancers, Rac proteins are hyperactive due to the upregulation of their upstream effectors, guanine nucleotide exchange factors (GEFs). Therefore, we have characterized a series of small molecules designed to inhibit the interaction of Rac and Cdc42 with their GEFs. We reported the development of EHop-016, a small molecule compound, which inhibits Rac1 and Rac3 activities of metastatic cancer cells with an IC₅₀ of 1 μM, and blocks the interaction of Rac with the oncogene and Rac and Cdc42 GEF Vav. EHop-016 also inhibits the activity of Cdc42 at higher concentrations, the Rac downstream effector p21-activated kinase (PAK), lamellipodia extension, migration, and viability of metastatic breast cancer cells. In this study, we further investigated the in vitro and in vivo action of EHop-016 in metastatic cancer, and report that EHop-016 inhibits the Rac activities of breast and prostate cancer cells, and human umbilical vein endothelial cells (HUVEC). EHop-016 may inhibit metastatic cancer cell viability by downregulating Akt and Jun kinase activities and c-Myc and Cyclin D expression, as well as increasing caspase 3/7 activities. Next, we tested the efficacy of EHop-016 in a nude mouse model of experimental metastasis, where female athymic nude mice bearing mammary fat pad tumors established from GFP-MDA-MB-435 cells were treated with 0, 5, 10, 25, or 40 mg/kg body weight (BW) EHop-016. Tumor growth and metastasis were quantified using fluorescence in vivo image analysis. We found that at 25 mg/kg BW, EHop-016 significantly reduces mammary fat pad tumor growth, metastasis, and angiogenesis. As quantified by ultra high pressure liquid chromatography (UPLC)/ mass spectrometry (MS/MS), EHop-016 was detectable in the plasma of nude mice at 17-23 ng/mL levels at 12 h following intraperitoneal (i.p.) administration of 10-25 mg/kg BW EHop-016. The EHop-016 mediated inhibition of angiogenesis in vivo was confirmed by in vitro tube formation assays of HUVECs. In conclusion, EHop-016 has potential as an anticancer compound to inhibit cancer progression

via multiple Rac-directed mechanisms. Moreover, EHop-016 is a useful tool to study Rac function in human disease in vitro and in vivo.

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Board Number: B420

Inhibition of thromboxane pathway, a promising new treatment against lung cancer.

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The efficacy of available treatments for the majority of lung cancer is still far from satisfactory, mainly due to lack of good therapeutic targets or severe side-effects. Recently, thromboxane (TX) pathway has been demonstrated to play a role in lung cancer development. Since the TX pathway is downstream of cyclooxygenase (COX), the inhibition of relevant targets in the TX pathway can avoid affecting the entire COX system, and thus improve the specificity of the treatment and reduce side-effects. In this study, we tested the TX inhibitor, and the TX receptor (TXR) blocker in lung cancer cells to check their effects on tumor cell proliferation, growth and apoptosis. A smoking carcinogen NNK-induced mouse lung tumor model was established to determine the effect of these inhibitors on the growth of lung tumor in vivo, and to examine the possible toxic effects of these treatments on liver and renal functions. Our results showed that TX was able to function as a critical mediator for tumor-promoting effects of COX-2 in lung cancer. Both TX specific inhibitors and TXR blockers could completely block NNK-mediated cell proliferation via inducing apoptosis. TXR agonist U46619 reconstituted a near full proliferative response to NNK when TXS was inhibited, affirming the role of TXR in tumor cell proliferation and growth. However, the dual TX inhibitor BM567 which inhibits both TX and TXR did not offer better suppression, compared with single TX inhibitor or TXR blocker, suggesting that TX and TXR were likely to function in a same channel to promote the tumor growth. The lung tumor model showed that the TX inhibitor led to the significant arrest of the tumor growth. Furthermore, TX inhibitors neither affected the daily activity of mice, nor caused damages to liver and kidney. In conclusion, the inhibition of TX pathway can lead to the significant suppression of lung tumor in vitro and in vivo without producing significant side-effects.

P286**Board Number: B421****Anti-Mullerian Hormone (AMH) and its Receptor AMHRII provide a TGF- β -Associated Pro-Survival Signaling Loop in Lung Cancer.**T.N. Beck¹, E. Nicolas², Y. Zhou³, I. Serebriiskii⁴, E.A. Golemis⁴;¹Molecular and Cell Biology and Genetics, Drexel University/Fox Chase Cancer Center, Philadelphia, PA,²Genotyping and Real-Time PCR, Fox Chase Cancer Center, Philadelphia, PA, ³Epidemiology andBiostatistics, Fox Chase Cancer Center, Philadelphia, PA, ⁴Developmental Therapeutics Program, Fox Chase Cancer Center, Philadelphia, PA

Signaling changes associated with epithelial-mesenchymal transition (EMT) affect prognosis and response to survival in lung cancer. The molecular chaperone heat shock protein 90 (HSP90) supports the activity of many protein clients associated with lung cancer pathogenesis including ERBB2, MET, BRAF and TGFBR1. Because of the dependence of cancer cells on HSP90 function, HSP90 inhibitors such as ganetespib selectively kill cancer cells, and are showing promising activity in clinical trials. We used RNAi screening to identify genes influencing lung cancer cell resistance to ganetespib. Depletion of Anti-Mullerian Hormone (AMH) and its receptor, AMH receptor type II (AMHR2), strongly sensitized cells to ganetespib in three RAS-mutated and one EML4-ALK expressing lung cancer cell lines. This was surprising, as neither the expression of AMH nor its receptor has ever been reported in lung cancer. Instead, almost all studies of AMH to date have focused on its role in differentiation of gonadal tissue, where it activates a cell surface receptor upstream of the SMAD genes, which are common effectors in the lung cancer-relevant TGF- β cascade, which controls EMT. Using multiple approaches, we have experimentally confirmed the expression of AMH and surface expression of AMHR2 in lung cancer cell lines, and mining of public databanks suggests that both proteins are expressed in a subset of lung cancer samples and cell lines. Surprisingly, we observed that depletion of AMH and AMHR2 induced EMT-like features, including downregulation of cadherins and assumption of a mesenchymal morphology. Conversely, ganetespib treatment increased epithelial features. These findings suggest that resistance to HSP90 inhibition may differ from resistance to traditional chemotherapeutics. In line with this hypothesis, we found that depletion of AMH and AMHR2 did not sensitize cells to cisplatin, while depletion of E-cadherin sensitized cells to HSP90 inhibition. Mechanistically, AMH and AMHR2 depletion depressed activity of NF- κ B and AKT, both critical EMT and survival regulators. These results for the first time indicate the presence of an AMH-AMHR2-NF κ B-AKT pro-survival signaling axis of therapeutic relevance in lung cancer. Further, our results also suggest that AMH, AMHR2 and EMT-associated proteins may serve as biomarkers to predict resistance to HSP90 inhibitors, and that it may be more beneficial to prime lung tumors with HSP90 inhibitors, to induce MET and decrease survival signaling, prior to treatment with chemotherapy.

P287**Board Number: B422****Molecular mechanisms underlying lung cancer progression.**J. Hooda¹, D. Cadinu¹, M. Alam², A. Shah², T.M. Cao², R. Brekken³, L. Zhang¹;¹Molecular and Cell Biology, Univ Texas-Dallas, Richardson, TX, ²Molecular and Cell Biology, University of Texas at Dallas, Richardson, TX, ³Division of Surgical Oncology, University of Texas Southwestern Medical Center, Dallas, TX

Altered metabolism is a hallmark of the transformed state in cancer progression. Recent studies have uncovered a plethora of metabolic alterations in cancer cells. One important finding is that mitochondrial respiration is amplified in certain human cancer cells. Recently, using a matched pair of cell lines representing normal nonmalignant HBEC30KT and non-small-cell lung cancer (NSCLC) HCC4017 cells developed from the same patient, we identified metabolic changes linked with the transformation of normal to cancer cells. We found that oxygen consumption and heme synthesis were intensified significantly in lung cancer cells, compared to the normal cells. Furthermore, the levels of heme uptake proteins and oxygen-utilizing hemoproteins were dramatically increased in cancer cells and xenograft tumors. Interestingly, we found that inhibition of heme synthesis or mitochondrial function preferentially suppressed cancer cell proliferation, colony formation and cell migration. These results demonstrated that heme availability is significantly increased in cancer cells and tumors, which leads to elevated production of hemoproteins, resulting in intensified oxygen consumption and cellular energy production for fueling cancer cell progression. Further, we also found that lowering heme availability sensitized NSCLC cells to paclitaxel. We measured the oxygen consumption of 6 NSCLC cell lines in the absence of 3 key metabolites individually and found that the levels of oxygen consumption increased in all the cell lines in the absence of glucose, while it decreased in the absence of glycine or glutamine in 4 of the cell lines. Experiments are currently underway to elucidate the roles of heme in cancer cell bioenergetics and function under different metabolic conditions.

P288**Board Number: B423****Antitumor studies of Modified L- Penetratin Peptide as a molecule targeting E2F in Prostate Cancer.**T. Shaik¹, N. Bansal¹, N.J. Farley¹, J. Kerrigan², E.E. Abali³, D. Banerjee¹, J.R. Bertino¹;¹Department of Medicine and Pharmacology, Rutgers Cancer Institute of New Jersey, New Brunswick, NJ, ²Department of Cancer Pharmacology and Preclinical Therapeutics, Rutgers Cancer Institute of New Jersey, New Brunswick, NJ, ³Department of Biochemistry and Molecular Biology, Rutgers Robert Wood Johnson Medical School, Piscataway, NJ

BACKGROUND: Mutation or inactivation of the retinoblastoma protein (pRB) is frequently involved in castrate resistant prostate cancer tumorigenesis resulting in overexpression/deregulation of E2F activity. E2F1-3a overexpression induces genes involved in DNA synthesis and leads to abnormal cellular

proliferation, tumor growth and invasion. Therefore, inhibiting the overexpression of one or more activating E2Fs is a recognized target in cancer therapeutics. In our previous studies we showed that a novel penetratin coupled 7-mer peptide (PEP), bound tightly to an immobilized consensus E2F1 promoter sequence, was cytotoxic to many malignant cell lines including Burkitt lymphoma cells, pRB negative small cell lung cancer cells and DU145 prostate cancer cells. Furthermore, treatment of tumor xenografts of DU145 tumors propagated in mice with a PEGylated liposome encapsulated PEP caused tumor regression; but tumor growth resumed when the drug was stopped.

OBJECTIVE: As the PEP was unstable in serum, we examined the antitumor activity and stability of two different modified penetratin peptides – 1. D- Arg penetratin peptide (substituting L- Arginine with D- Arginine in the peptide sequence); 2. N-acetylated, C-methylated penetratin peptide (end capping the peptide).

METHODS: DU145 (prostate cancer), H196 and H82 (small cell lung cancer) cells were used. To compare the efficacy of the peptides, we tested the IC50s of peptides at different time points in various cell lines by MTS assay. Stability tests were performed by incubating the peptides in RPMI media containing 10% FBS for 24 hours and then measuring cell viability using MTS assay. Drug combination experiment results were analyzed using the combination index method developed by Chou and Talalay.

RESULTS: D- Arg penetratin peptide was more potent compared to L- Arg penetratin peptide and it was found to be more resistant to degradation by serum proteases than the L- form. The other modified form, N-acetylated, C-methylated PEP was marginally more effective than the unmodified PEP. Drug combination studies showed that D- Arg penetratin peptide in combination with docetaxel, caused additive cytotoxicity against DU 145 cells. Our findings validate D- Arg peptide, an inhibitor of E2F1, as a drug for targeted molecular therapy of prostate cancers with elevated levels of activated E2F's; and encourage future modifications of the peptide that include all D- amino acid substituted peptide.

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Board Number: B424

Study of the Effects of Lysophosphatidic Acid on Lymphatic Metastasis of Prostate Cancer.

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Clinical evidences suggested that lymphangiogenesis and lymphatic metastasis are important processes during the progression of prostate cancer. Vascular endothelial growth factor (VEGF)-C was shown to be a key regulator during these processes. Our previous studies demonstrated that lysophosphatidic acid (LPA) enhances VEGF-C expression in human endothelial cells. However, the effects of LPA on VEGF-C expression in prostate cancer remain elusive. We demonstrated that LPA enhanced VEGF-C expression

in three different human prostate cancer cell lines. Moreover, the enhancement effect is mediated through activating LPA1/3, reactive oxygen species (ROS) generation, and action of lens epithelium derived growth factor (LEDGF). Furthermore, PC-3 cells were injected in nude mice followed by LPA1/3 antagonist Ki16425 treatment to evaluate the effects of chemical inhibition on LPA receptor in blocking lymphatic metastasis of prostate cancer. We monitored the endothelial and lymphatic markers expression in the xenografts by immunochemistry stainings. We demonstrated that Ki16425 treatment reduced lymphatic markers expression in tumors. These results may potentially lead to future strategies for preventing lymphatic metastasis of prostate cancer.

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Board Number: B425

Systematic interrogation of druggable pathways in pancreatic adenocarcinoma.

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Despite significant advances in the development of cancer treatment in the last 20 years the best available therapies have marginally improved the survival rate of treated pancreatic cancer patients (from 3% to 6% five-year survival), making it still one of the deadliest malignancies. Single standard of care cytotoxic and targeted therapies demonstrate little to no effect on patient survival. Even though we now understand some of the main mutated genetic factors present in pancreatic tumors, their role in drug response remains poorly understood. To identify drug-sensitizing genetic targets in cancer cells we have developed a high-throughput screening platform. In this methodology, pooled lentiviral libraries containing hundreds of knockout constructs specific for cancer-related genes are used to infect cancer cells. By treating the infected populations with therapeutic agents we are able to identify genetic targets that when inhibited synergize with existing drugs to cause increased cell death. We piloted this type of screening platform in HCT-116 cells, a KRAS mutant, MEK inhibitor resistant colorectal cancer cell line, using the MEK-inhibitor AZD-2644 and a pooled shRNA library. This study identified 4-7 target genes that when knocked down sensitized the previously resistant HCT-116 cells to AZD-2644. To conduct a more robust and representative sensitization screen we developed a lentiviral CRISPR-Cas9 library. By performing screens in a panel of pancreatic cancer cell lines, we have identified both cell line dependencies and sensitizers to targeted and cytotoxic chemotherapies. The findings from these screens provide potential mechanistic insights into the key survival signaling programs in pancreatic cancer as well as new therapeutic strategies.

P291**Board Number: B426****Treatment of pancreatic cancer cell lines with high-affinity galectin-3 inhibitor further confirms novel intracellular function of galectin-3 and implications in anti-cancer therapy.**J. Stegmayr^{1,2}, G. Sharma¹, M. Carlsson^{1,3}, S. Oredsson², U.J. Nilsson⁴, H. Leffler¹;¹Department of Laboratory Medicine, Lund University, Lund, Sweden, ²Department of Biology, Lund University, Lund, Sweden, ³Department of Cellular and Molecular Medicine, University of Copenhagen, Copenhagen, Denmark, ⁴Department of Chemistry, Lund University, Lund, Sweden

Galectin-3, a protein with the affinity for β -galactosides, has been implicated in a number of cancer-related functions. In pancreatic cancer cell lines, for example, galectin-3 knockdown results in decreased proliferation and migration, and increased gemcitabine-induced apoptosis. Surprisingly, treatment of the pancreatic cancer cell lines MIA PaCa-2 and PanC-1 with a novel high-affinity (K_d in the low nM range) galectin-3 antagonist, bis-{3,3'-deoxy-3,3'-[4-(3-fluorophenyl)-1H-1,2,3-triazol-1-yl]}-1,1'-sulfanediyl-di- β -D-galactopyranoside, had no effect on cell proliferation or gemcitabine-induced cell death. The lack of effect was not due to insufficient intracellular accessibility of the galectin-3 antagonist, because it efficiently inhibited accumulation of galectin-3 around glycy-L-phenylalanine 2-naphthylamide (GPN)-damaged lysosomes, a phenomenon known to depend on the carbohydrate-binding activity of cytosolic galectin-3. The inhibition of galectin-3 accumulation around GPN-damaged lysosomes by the antagonist was seen to be both time- and dose-dependent, as investigated by quantifying galectin-3 puncta formation using immunocytochemistry and confocal microscopy. This indirectly suggests that the proliferation promoting and apoptosis inhibiting effects of galectin-3 may not require its carbohydrate-binding activity. Additionally, treatment of both of the pancreatic cancer cell lines with GPN in combination with the galectin-3 antagonist led to an increased reduction in cell viability compared to treatment with GPN alone, suggesting a protective role of galectin-3 after lysosomal insult. Taken together these results further endorse a novel intracellular function of cytosolic galectin-3, which is to accumulate around and protect lysosomes upon damage, which in turn may render galectin-3 antagonist interesting agents in anti-cancer therapy.

P292**Board Number: B427****Pharmacological activation of myosin II to correct pancreatic cancer cell mechanics.**A. Surcel¹, Q. Zhu², E.S. Schiffhauer¹, H. West-Foyle¹, R.A. Anders², D. Robinson^{1,3};¹Department of Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD, ²Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD, ³Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD

Pancreatic ductal adenocarcinoma (PDAC) annually affects 44,000 people in the U.S. and has an abysmal five-year survival rate of around 6%, which is nearly unchanged over the past 40 years. Pharmacological strategies for treating cancer have primarily focused on inhibiting cell growth through specific genetic pathways, which typically either fail to abolish the disease or lead to compensatory regulatory changes and subsequently, to drug resistance. Importantly, alterations in mechanical properties are a common feature of cancer cells, yet targeting cell mechanics remains an under-utilized approach for drug development.

Here we develop a system for targeting cell mechanics for the discovery of novel therapeutics. We designed a live-cell, high-throughput chemical screen to identify mechanical modulators in *Dictyostelium discoideum*. We characterized 4-hydroxyacetophenone (4-HAP), which increases the cellular cortical tension by enhancing the cortical localization of the mechanoenzyme myosin II, independent of myosin heavy-chain phosphorylation regulation. To shift cell mechanics, 4-HAP requires myosin II, including its full power stroke. We further establish that changes in key cytoskeletal protein distributions correlate with the changes in the biomechanical profile of PDAC progression. In addition to actin-crosslinkers, we detect that non-muscle myosin II distributions vary across PDAC states: specifically myosin IIA increases, myosin IIB decreases, and myosin IIC increases in metastatic cells. We further demonstrate that invasive pancreatic cancer cells are more deformable than normal pancreatic ductal epithelial cells, a mechanical profile that was partially corrected with 4-HAP. Tests of 4-HAP in mouse models of metastatic pancreatic disease are underway. Overall, 4-HAP modifies non-muscle myosin II-based cell mechanics across phyla and disease states and provides proof-of-concept that cell mechanics offer a rich drug target space, allowing for possible corrective modulation of tumor cell behavior.

P293**Board Number: B428****Modulation of the Unfolded Protein Response in Multiple Myeloma.**J. Thomas¹, y. argon²;¹children's hospital of philadelphia, Philadelphia, PA, ²children's hospital of philadelphia, philadelphia, PA

Multiple Myeloma (MM) is the second most common hematological malignancy in the U.S.A. with over 20,000 new cases reported each year and a life expectancy in the range of 3-6 years. MM is

characterized by increased numbers of bone marrow plasma cells that secrete copious quantities of antibodies. The IRE1 kinase/ribonuclease arm of the unfolded protein response (UPR) is activated in MM cells and is necessary for their ability to sustain antibody production. The UPR is initially cytoprotective in that it alleviates ER stress that results from this sustained antibody production. However, IRE1 activity can be modulated to promote apoptosis through both the JNK and regulated IRE1-dependent decay of mRNAs (RIDD) pathways. The clinical proteasome inhibitor Bortezomib (Bz) is the most common drug used to treat MM. Since IRE1 activity is transient, the progression of MM that is characterized by Bz resistant clones under selection pressure may occur due to eventual attenuation of IRE1. Therefore, a single-cell assay method to detect and tract IRE1 activity in Bz resistant clones will be invaluable in understanding how to target therapy resistance. Our lab is developing a fluorescent protein reporter that will be able to identify individual MM cells and determine if a correlation exists between Bz resistance and attenuation of IRE1 activity. Our way IRE1 activity can be attenuated in Bz resistant clones is through a new modulator of IRE1 that was discovered in the Argon lab: the ER enzyme PDIA6, which binds to IRE1 and terminates its XBP1s activity. We are interested in determining if knockdown of PDIA6 in multiple myeloma will increase sensitivity to Bz due to prolonged activation of IRE1. The ability to identify Bz resistant clones, and modulate their IRE1 activity could be a novel therapeutic approach to treat MM.

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Board Number: B429

Extracellular matrix mechanics causes systematic variation in cancer cell proliferation and responses to drugs against myeloid leukemias.

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Emerging evidence pinpoints the bone marrow (BM) milieu as a cell extrinsic contributor to the pathophysiology and chemotherapeutic resistance of cancer. Cancer progression is frequently associated with profound alterations in the BM microenvironment by collagen deposition that leads to fibrosis and changes in mechanical properties of the matrix. While the relevance of extracellular matrix mechanics in solid tumors has been investigated, its role in causing the progression and drug resistance of blood cancers or “liquid tumors” remains unclear. Myeloid leukemias consist of a number of disease subtypes defined by distinct genetic mutations and constitutive activation of signaling pathways. Some of these pathways, including Protein Kinase B (AKT), are involved in mechanotransduction, and hence may influence sensitivities of leukemia cells to matrix stiffness. The study is thus based on the hypothesis that matrix stiffness differentially regulates cell proliferation of myeloid leukemia subtypes. By culturing cells in an engineered 3D hydrogel system with tunable stiffness and independent control of ligand density, we demonstrate distinct *ex vivo* cell proliferation profiles and colony growth patterns in response to matrix mechanics for different leukemia subtypes, including human acute and chronic myeloid leukemias (AML and CML). Importantly, *ex vivo* growth of leukemia cells in this system reflects

in vivo growth observed in a xenograft human myeloid sarcoma model, which is generated by subcutaneously implanting leukemia cells encapsulated in the hydrogel with different stiffness. Varying ligand density further modulates leukemia cell proliferation. Interestingly, CML cells are resistant to an AKT inhibitor in the 3D hydrogel compared to the standard plastic culture, while AML cells are generally responsive to the treatment. Further studies reveal that CML cells in the viscous matrix are more resistant to existing drugs (e.g. Imatinib) than those in the solid matrix. Drug screening studies show that different small molecules can be classified based on pharmacodynamics and cell proliferation kinetics profiles of CML cells in different matrix mechanics. Using this approach, we have identified a subset of drugs that may be useful to inhibit CML cell proliferation regardless of matrix mechanics. The results suggest a potential approach to design a drug regimen to reduce the probability of minimal residual disease that could be present in different microenvironments, which can be tailored to individual blood cancer subtypes prior to administration.

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Board Number: B430

Blockage of prion protein-HOP engagement impairs glioblastoma growth and improves overall survival.

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Glioblastomas (GBMs) are resistant to current therapy protocols and identification of molecules that target these tumors is crucial. Interaction of secreted heat-shock protein 70 (Hsp70)–Hsp90-organizing protein (HOP) with cellular prion protein (PrPC) triggers a large number of trophic effects in the nervous system. We found that both PrPC and HOP are highly expressed in human GBM samples relative to non-tumoral tissue or astrocytoma grades I–III. High levels of PrPC and HOP were associated with greater GBM proliferation and lower patient survival. HOP–PrPC binding increased GBM proliferation *in vitro* via phosphatidylinositol 3-kinase and extracellular-signal-regulated kinase pathways, and a HOP peptide mimicking the PrPC binding site (HOP230–245) abrogates this effect. PrPC knockdown impaired tumor growth and increased survival of mice with tumors. In mice, intratumor delivery of HOP230–245 peptide impaired proliferation and promoted apoptosis of GBM cells. In addition, treatment with HOP230–245 peptide inhibited tumor growth, maintained cognitive performance and improved survival. Thus, together, the present results indicate that interfering with PrPC–HOP engagement is a promising approach for GBM therapy.

P296**Board Number: B431****Effect of MiR-26a on Human Malignant Melanoma Cells.**G. Yang¹, G. Gao¹, J. Jiang¹, Y. Yang¹;¹Emporia State University, Emporia, KS

Objective: The goal of this study is to examine the effect of Let-7a on the proliferation and invasiveness of human malignant melanoma cells and identify the gene(s) that Let-7a targets to affect the growth and metastasis of melanoma cells. **Methods:** The effect of Let-7a on proliferation, migration and invasion was measured by cell viability assay and Wounding healing assays, and Transwell migration assay, respectively. The expressions of MITF, and MAP4K3 genes at protein level were measured by Western blot. Cell cycle progression was determined by flow cytometer. **Results:** Let-7a displayed a significant ($p < 0.05$) inhibitory effect on the proliferation, migration and invasion on SKMEL-28 and WM1552C human melanoma cell lines. In addition, Let-7a induced cell cycle arrest at G1 phase. MITF gene was down-regulated by Let-7a in melanoma cells. **Conclusion:** Let-7a inhibited the growth, migration and invasion, also induced cell cycle arrest at G1 phase in human malignant melanoma cells. The expression of MITF was suppressed by Let-7a in SKMEL-28 and WM1552C melanoma cell lines. Taken together, these data suggests Let-7a act as tumor suppressors in melanoma cells and have the potential to function as a novel therapeutic small molecule against human malignant melanoma.

P297**Board Number: B432****P53 interactome and β -tubulin isotypes as biomarkers for head and neck squamous cell carcinoma.**S. Lobert¹, M.E. Graichen¹, R.D. Hamilton¹, K.T. Pitman², M.R. Garrett¹, C. Hicks¹, T. Koganti¹;¹Univ Mississippi Med Ctr, Jackson, MS, ²Banner MD Anderson Cancer Center, Gilbert, AZ

In 2014, more than 40,000 people in the United States will be diagnosed with head and neck squamous cell cancer (HNSCC) and nearly 8,400 people will die of the disease (www.cancer.org/acs/groups). A reduction in the activity of the tumor suppressor p53 in large numbers of patients with HNSCC is associated with HPV infection and HPV infection is often linked to better patient outcomes; however, little is known regarding molecular targets that might lead to better therapies and improved outcomes for these patients. The incorporation of taxanes into the standard cisplatin/5-fluouracil initial chemotherapy for HNSCC has been associated with improved response rate and survival. Taxanes target the beta-subunit of the tubulin heterodimers, the major protein in microtubules, and halt cell division at G2/M phase. Both laboratory and clinical research suggest a link between beta-tubulin expression and cancer patient survival, indicating that patterns of expression for beta-tubulin isotypes along with activity of tumor suppressors such as p53 or micro-RNAs could be useful prognostic biomarkers and could suggest therapeutic targets. In this study we used quantitative real time PCR and microarray analysis to compare HNSCC samples and associated normal tissues from 33 patients. We measured

mRNA for six beta-tubulin isotypes: classes I, IIA, IIB, III, IVB and V, using quantitative real time PCR and in addition, used microarray analysis to explore the expression of > 30,000 genes. We found reduced activity of the p53 interactome is associated with progressive disease at two years and elevated beta-tubulin class III was linked to a trend for disease-free survival over the same time period.

P298

Board Number: B433

Characterization of particle-endothelium interaction using particles functionalized with dual antibodies in a complex synthetic microvascular network.

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The interaction between leukocytes and receptors on the endothelium play a key role in the early response to tissue injury. Research has shown that drug carrier particle rolling velocity increases dramatically with the increase of carrier size which affects their final attachment efficiency. The objective of this study is to characterize the rolling and adhesion profile of different size functionalized particles in a synthetic microvascular network (SMN).

Functionalized particles were prepared by coating their surfaces with different ratios of antibodies against adhesion molecules ICAM-1 and E-selectin. Our modified Geographic Information System (GIS) approach was used to digitize the microvascular networks and generate the SMN on PDMS. The rolling velocity and adhesion profile of functionalized 2 & 10 μm particles was quantified in the SMN pre-coated with TNF- α activated human umbilical vein endothelial cells under shear conditions from 0 – 280 sec^{-1} .

Single antibody coated 10 μm particles roll at a higher speed than its 2 μm counterparts. Particle adhesion increased significantly with decreasing shear in SMNs. Increasing particle size from 2 μm to 10 μm did not change its binding efficiency significantly in SMNs, which is different from what have been reported in the traditional parallel plate flow channel.

Particles functionalized with both a rolling molecule and an adhesion particle can more realistically mimic the leukocyte-endothelium interaction in a SMN. These findings have important implications for better understanding the mechanisms behind inflammatory pathologies and for optimizing the design of carriers for targeted drug delivery.

P299**Board Number: B434****The Effect of Androgen on Cell Proliferation in Prostate Cancer In Vitro Models.**S. Turner¹, D. Lamb²;¹Department of Biology, Prairie View AM University, Prairie View, TX, ²Departments of Urology and Molecular Cellular Biology. , Baylor College of Medicine, Houston, TX

Prostate Cancer (PCa) is the second most common cancer amongst older men, and one of six men is diagnosed with PCa in their lifetime. Of note, one in 30 men diagnosed with PCa will succumb to their disease. African-American males and men over the age of 50 are at an increased risk. PCa has a multifactorial etiology. Typically, PCa is a slow growing cancer; most men will never know they have the disease. During the early stages of PCa, men experience few to no symptoms. Currently, 85 percent of American men are diagnosed with PCa in its early stages, which are treatable, allowing these men to carry out a moderately normal life. Unlike other cancers, victims of PCa will die due unrelated causes. The growth of PCa is androgen responsive. The effect of androgens, such as testosterone (T) and dihydrotestosterone (DHT) is mediated through its interactions with the androgen receptor (AR), which plays crucial roles in cell proliferation and differentiation,(processes important for prostate development and PCa, respectively). AR activity is associated with disease progression and therefore, the men with advanced disease are treated with androgen ablation therapy. For this study, human PCa cell lines, LNCaP cells and PC3, which lack androgen receptors, were used. LNCaP are androgen responsive while PC3 are androgen insensitive. R1881 is a synthetic androgen used to study the effects of androgen action. Studies in LNCaP cells show a bimodal effect of androgens on cell proliferation. Low concentrations (<10nM R1881) drive proliferation and high concentrations (>10nM) inhibit proliferation. The purpose of this project was to examine the effect of several concentrations of R1881 on cell proliferation in LNCaP and PC3 cells using several cell quantification techniques. These included counting using the vi-cell, a trypan blue cell analyzer, a Hemocytometer, and a WST (tetrazolium salt viability) assay to determine cell proliferation for a range of days. It was hypothesized that the growth of LNCaP cells at higher concentrations of R1881 would be inhibited; however PC3 cells would not be inhibited. Using the aforementioned techniques, it was found that more than 10nM of R1881 inhibits cell proliferation in LNCaP cells, and less than 10nM of R1881 stimulated cellular proliferation in these cells, indicating these are sensitive to androgens. Furthermore, no effects were seen on PC3 cellular proliferation with lower concentrations of R1881. In conclusion, androgens working through the androgen receptor exhibit a dosage sensitive action on LNCaP growth *in vitro*.

P300**Board Number: B435****A Cure for Prostate Cancer is Possible.**A.A. Sinha¹;¹Univ. of Minnesota , V A Med Ctr Genetics, Cell Biology , Minneapolis, MN

Androgen ablation/deprivation by castration, chemical castration by diethylstilbestrol (DES), or by a variety of FDA-approved drugs has provided palliative treatment for prostate cancer (PCa). Our objective was to find the reasons for the treatment failures. To do this we studied untreated and DES treated biopsies for localization of androgen (AR) and estrogen (ER) receptors using antibodies and immunogold techniques. We examined biopsies of eight DES treated (ranging from 37 days to 18 years) and 18 untreated PCa patients with Gleason histological score 7 and higher grade tumors. Epon-embedded thick sections prepared with a Reichert-Jung microtome were stained with methylene blue. We used two types of androgen receptor antibodies (AR, N-20, Santa Cruz Biotechnol., CA, and anti-AR-V-7 (Precision antibody, A& G Pharmaceutical, Inc. Columbia, MD) and estrogen receptors (ERbeta, H-150, Santa Cruz). Receptors were localized using immunogold techniques and protein A conjugated to 15nm gold particles enhanced with silver. Androgen and estrogen receptors were localized in acini and invasive cells of untreated and DES treated sections. At the electron microscopic level, estrogen receptors localized on the nuclear membranes and the adjacent heterochromatin, but not in nucleoli. Analysis of data indicated that androgen ablation was incomplete since androgen receptors were present in both treated and untreated samples. This led to review of the steroid biosynthetic pathway. This pathway utilizes one or more specific enzymes to catalyze, for example, cholesterol into pregnenolone followed by enzymes that leads to production of progesterone then to testosterone and finally to estrogen. Androgen ablation, undoubtedly, decreases the supply of testosterone in prostate, but it does not inhibit the production of the enzymes in the steroid pathway. Enzymes involved in catalyzing progesterone to testosterone ought to be inhibited for androgen ablation. Since progesterone, testosterone and estrogen and their receptors are found in malignant prostate, inhibitions of enzymes are essential for suppressing production and supply of gonadal steroid hormones to cancer cells. Presence of estrogen receptors in untreated and DES treated PCa indicates that androgen ablation was bypassed in the prostate. Our study indicates that in addition to androgen ablation, we must also inhibit enzymes that catalyze progesterone for the production of testosterone. In conclusion, we suggest that inhibition of enzymes catalyzing cholesterol to pregnenolone have the potential of not only significantly improving treatment of PCa, but also of breast cancer and other hormone-dependent cancers.

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P301**Board Number: B436****CuS + Graphene Oxide + Folate nanocompound = X factor in Photothermal Therapy.**D.M. Peterson¹, D. Gilbert², S. Henderson-Jones³, A. Novelo², K. Manns², L.E. Carson⁴, A. Oki⁵, E.C. Regisford²;¹Biology, Prairie View AM University, Prairie View, TX, ²Department of Biology, Prairie View AM University, Prairie View, TX, ³Prairie View AM University, Prairie View, TX, ⁴Cooperative Agricultural Research Center, Prairie View AM University, Prairie View, TX, ⁵Chemistry Department, Prairie View AM University, Praire View, TX

Photothermal Therapy (PTT) is a novel approach at cellular irradiation. PTT manipulates electromagnetic radiation of select particles at an excitable wavelength. Inferred, radiofrequency, microwaves, and ultrasonic wavelengths have all been used to elicit excitation. Excitation, due to photons transfer of electromagnetic energy to selected particles, leads to release of heat energy via vibration. Heat builds up in the cell causing cell membrane lysis leading to cellular death. Copper sulfide (CuS) a black colloid, is used as irradiant. To transport CuS into the cell, Graphene Oxide a single layer form of graphite, is used as its vehicle. In order to deliver CuS + Graphene Oxide to a targeted cancer cell, a known cancer biomarker must be identified. Many cancer cell lines increase up-regulation of the folic acid receptors, these receptors bind to Folate as its ligand. Thus, this study takes advantage of folate's ability to act as ligand to bind the CuS + Graphene Oxide to cell surface and induce its cellular uptake via endocytosis. Once the compound is inside the cell, a practitioner may irradiate the targeted area with an external light source. This targeted approach will only kill cells that have up-taken the CuS + Graphene Oxide+ Folate compound. Finding the optimal concentration of the nanoparticle compounds is paramount. SKOV-3, Hela, A549, and hFOB-1 cell lines were used. Cells were treated with [50ng/ml], [100ng/ml], [200 ng/ml], [400 ng/ml], and [800 ng/ml] concentrations of CuS, Folate, Graphene oxide, Graphene oxide + Folate, CuS + Folate, and Graphene oxide + Folate + CuS, and an untreated control group. An assay , specifically, 3-(4,5-dimethylthiazolyl-2)-2, 5 diphenyltetrazolium bromide (MTT) assay was then used to measure cell viability and proliferation.

P302**Board Number: B437****Dihydrocucurbitacin B potently induces apoptosis in acute leukemic cell lines of B, T and promyelocytic origin.**M. Martlock¹, T. Laakko Train¹;¹Department of Biology, Elon University, Elon, NC

Leukemia is responsible for 25% of cancer diagnoses in patients under age 20. While major advances have been made in treatments, effectiveness varies depending on the type of leukemia and there is still

significant mortality. Cucurbitacins are a group of compounds derived from plants that have been shown to have anti-inflammatory and anti-tumor effects. The cucurbitacin dihydrocucurbitacin B (DHB) is a compound found in *Cayaponia tayuya*, a Brazilian tree thought to have anti-inflammatory properties. Previous work from our lab has shown that treatment of leukemic cells with DHB or *tayuya* extract causes an increase in apoptosis in an acute T-cell leukemia cell line (Jurkat). To determine whether DHB had similar effects on other types of leukemic cells, additional cell lines were investigated. Cells were cultured with or without DHB at concentrations ranging from 0.25-25 μM from six to 24 hours. Apoptosis was detected by Annexin V and propidium iodide (PI) staining of cells followed by flow cytometric analysis. Significant increases in Annexin V+/PI- (early and late apoptotic) cells were observed within hours of exposure to the lowest (0.25 μM) concentration tested. After 24 hours with 2.5 μM DHB, the mean percent of apoptotic cells significantly increased from 13.2% in controls to 44.6% (a 3.4-fold increase) in treated samples. To determine if this response was similar in different types of leukemic cells, the RAJI cell line (Burkitt B-cell lymphoma) and HL-60 cell line (an acute promyelocytic leukemia) were cultured with varying concentrations of DHB. Results similar to those observed in Jurkat cells were observed with RAJI cells; after 24 hours without or with 2.5 μM DHB, the average percent apoptosis increased nearly 3-fold from 11.5% in controls to 31.2%, respectively. Interestingly, the promyelocytic line, HL-60, showed a more dramatic increase in apoptosis under the same conditions. The average percent apoptosis increased nearly 20-fold, from 4.5% in controls to 89.0% in treated samples. This data indicates that DHB potently induces apoptosis in a variety of different leukemic cell types and cells of myeloid lineage had a much greater response than those of lymphoid origin. Future studies will investigate how primary immune cells respond to DHB and explore potential mechanisms for induction of apoptosis.

P303

Board Number: B438

POTENTIAL THERAPEUTIC STRATEGY FOR NON-MUSCLE INVASIVE BLADDER CANCER (NMIBC) TREATMENT: EFFECTS OF DOXORUBICIN AND CISPLATIN LOADED IN REDUCED GRAPHENE OXIDE ON THE PI3K AND PTEN REGULATION.

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Background: Bladder cancer is the most common malignancy of the urinary tract. The recommended first-line treatment for non-muscle invasive bladder cancer (NMIBC), following transurethral resection, is an induction course plus maintenance with intravesical *Bacillus Calmette-Guerin* (BCG). However, BCG immunotherapy causes undesirable effects, which contributes to treatment interruption besides increasing cancer index recurrence after treatment. In this context, graphene oxide (GO) and/or reduced graphene oxide (rGO) has attracted increasing interest in the field of biological detection, drug delivery,

and cancer therapies. Thus, the aims of this study were to characterize and to compare the antitumor effects of Doxorubicin (DOX) and Cisplatin (CIS) functionalized in rGO on NMIBC. **Methods:** The protocol to induce NMIBC was the following: twenty-five female Fischer 344 rats received 1.5 mg/kg dose of N-methyl-N-nitrosourea (MNU) intravesically (I.V.) and the 5 Control animals (Group 1) had received physiological saline. After MNU treatment, the Cancer group (Group 2) received the same treatment as Group 1; The Cancer+rGO group (Group 3) received 2.0 mg/mL dose of reduced graphene oxide; The Cancer+rGO+CIS group (Group 4) received 1.5 mg/kg dose of cisplatin; The Cancer+rGO+DOXO group (Group 5) received 3.0 mg/mL dose of doxorubicin; The Cancer+rGO+CIS+DOXO group (Group 6) received the same treatment as Groups 3, 4 and 5; all treatments I.V. for 6 weeks. After 16 weeks, all bladders were collected for histopathological and immunohistochemical analysis. **Results:** The results demonstrated that 100% of animals from Group 2 showed carcinoma with invasion of the lamina propria (pT1). Similarly, the Groups 3 and 4 showed high-grade papillary carcinoma (pTa), carcinoma *in situ* (pTis) and pT1 in 60%, 20% and 20% of animals, respectively. The Group 5 showed pTis, pTa and low-grade intraurothelial neoplasia in 60%, 20% and 20% of animals, respectively. There was a better histological recovery in the Group 6, which showed 40% of flat hyperplasia, 40% of low-grade intraurothelial neoplasia and 20% of pTis. Intensified PI3K (Phosphatidylinositol-4,5-bisphosphate 3-kinase) immunoreactivities was observed in the Groups 2, 3, 4 and 5 when compared to Group 6. In contrast, the Groups 2, 3, 4 and 5 showed weak PTEN (Phosphatase and tensin homolog) immunoreactivities in relation to Groups 1 and 6. **Conclusions:** The tumor regression grades were correlated with increased PTEN and decreased PI3K reactivities in the Cancer+rGO+CIS+DOXO group. Taking together, the data suggest the great potential of reduced graphene oxide as carrier system of doxorubicin and cisplatin, and as nanoformulation for NMIBC.

P304

Board Number: B439

EVALUATION OF NEW HDACs INHIBITORS OVER TUMORAL AND NORMAL CELL LINES.

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HDAC inhibitors (HDACi) have been prove over diverse antitumoral effects such as cell growth inhibition, differentiation induction and apoptosis triggers¹. In this work we prove the antitumoral action of new HDACi previously synthetized with HDAC inhibition activity reported², over three tumoral cell lines: MCF-7, HepG2 and HeLa, as well as, two normal cells that normally present damage in the chemotherapy process like endothelial cells and kidney cells. HDACs are considered as one promising target in the development of new antitumoral agents, due to their actions on apoptosis, cell proliferation, metastasis and cell growth inhibition. Different HDACi have been reported as antitumoral compounds, between them there are the isoindoline analogues^{3y4}. In 2014 this group reported a series of insoindolines-2-sustituted as new HDACi, these compounds show a good HDAC inhibitory activity,

that is why in this work there was evaluated their antitumoral capacity over different tumoral cell lines from mammary cancer, hepatocarcinome and cervical uterin cancer. Due the importance of the adverse reactions in patients treated with several antitumoral agents, we considered of relevance to prove this new agents over possible toxic effects on turn over cells like endothelial, and cells usually very exposed to chemical agents due to elimination process as kidney cells. Our findings showed that all isoindolines evaluated produced growth inhibition of tumoral cells, specially compounds 1b, 1f and 2d. Whereas isoindolines 1a, 2a, 2b and 2c showed minor cytotoxicity on endothelial cells and kidney cells. By microscopy we observed the effects that denote a possible apoptotic pathway in tumoral cells. Therefore isoindolines-2-substituted can be used as possible antitumoral agents with low cytotoxicity in normal cells in the treatment of mammary cancer, hepatocarcinome and cervic uterine cancer.

Kirsten Grønbeek, Christoffer Hother and Peter A. Jones. Epigenetic changes in cancer, *Journal Compilation*, 115: 1039–1059, 2007.

Cynthia R. Trejo Muñoz, Elvia Mera Jiménez, Rodolfo Pinto Almazan, José Alfredo Díaz Gandarilla, José Correa Basurto, José G. Trujillo Ferrara, Christian Guerra Araiza, Patricia Talamás Rohana y Teresa Mancilla Percino. Effect of two of isoindolines over HDAC8 activity and expression. *Medicinal Chemistry Research* 23: 3227-3234, 2014.

Shoukou L., Chihiro S., Kiyoshi O., Motomu S., Satoko M., Mayumi S., Minoru Y., Yuichi H. y Hiroyuki M., Design, synthesis, and evaluation of isoindolinone-hydroxamic acid derivatives as histone deacetylase (HDAC) inhibitors, *Bioorganic and Medicinal Chemistry Letters*, 17: 4895-4900, 2007

Chihiro S., Takamuri N., Satoko M., Minoru Y., Yuichi H., Hiroyuki M., Design and synthesis of phtalidamide-type histone deacetylase inhibitors, *Bioorganic and Medicinal Chemistry Letters*, 15: 4427-4431, 2005

P305

Board Number: B440

Using confocal laser scanning microscopy to monitor targeted delivery of chemotherapeutic agents.

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OBJECTIVE: The objective of this study is to use confocal laser scanning microscopy (CLSM) to monitor drug uptake and movement within MDA-MB-231 breast cancer cells.

BACKGROUND: A favorable property of chemotherapeutic agents is the preferential delivery of cancer drug to the tumor tissue, lessening damage to healthy surrounding tissue. An experimental drug conjugate was used for this study and consisted of two components. Component A (A) acts as a delivery vector for cancer therapeutics, binding to LDL and entering the cell via the LDL-receptor pathway. Once the drug conjugate is in the cell, it moves into the lysosomes where the low pH environment cleaves off

drug component B (B). Drug component B is the cancer therapeutic doxorubicin that travels to the nucleus where it intercalates into the DNA to prevent transcription and eventually leads to cell death.

METHODS: Human breast cancer cell line, MDA-MB-231 cells were maintained in DMEM/1% Penicillin-Streptomycin/10% Fetal Bovine Serum. The cells were plated in poly-l-lysine coated Mattek dishes and incubated overnight. The drug was added to the cells followed by overnight incubation. After rinsing the cells, wheat germ agglutinin (WGA)-Alexa Fluor 488 was added, followed by fixing and cover slipping using Prolong® Gold containing DAPI. Samples were imaged on a Nikon A1Rsi CLSM at 405nm (DAPI, blue), 488nm (WGA, green), 561nm (B, orange) and 638nm (A, red). Each channel was acquired separately to avoid emission cross over between channels.

To demonstrate the movement of the drug to the lysosomes, the cells were incubated with the drug as above, stained with LysoTracker® and imaged live. In addition, cells were blocked with LDL/heparin and imaged live to prevent the uptake of the drug into the cells. In each case, negative controls were imaged in parallel.

RESULTS: Confocal laser scanning microscopy was utilized to: 1) image the drug incorporation into MDA-MB-231 breast cancer cells, 2) demonstrate the localization of the drug in the lysosomes and 3) show that the conjugate uptake is LDL-mediated.

CONCLUSION: We used confocal microscopy to demonstrate the uptake of the drug into the cell, movement to the lysosome, followed by the nucleus. We also verified the mechanism by blocking the uptake of the drug.

P306

Board Number: B441

The effects of vanadyl sulphate on rat glioma cells ultrastructure.

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Vanadium compounds were stated to have different effects on the cells as to be genotoxic, lipid peroxidative, erythrocyte hemolytic and effective in inhibition of neoplastic progression. One of the most popular vanadium salts is vanadyl sulphate that is investigated in different types of cells, also in vivo. In this study vanadyl sulphate was used with an aim to detect the effects of a vanadium compound on the rat glioma cells (C6) ultrastructure. The effects of vanadyl sulphate on glioma cell ultrastructure were investigated using a transmission electron microscopic assay. For this manner, C6 cells treated with IC50 concentration (100 µM) of vanadyl sulphate for 24 hours were fixed in glutaraldehyde (2,5% in 0,1 M phosphate buffer) at pH 7.4 and embedded in agar. Embedded samples were left in phosphate buffer at +4 °C, overnight and post fixed in osmium tetroxide. Post-fixed cells were dehydrated in graded ethanol: 70, 90, 96 and 100% than embedded in EPON 812 epoxy and left 48 hours in oven for polymerization. The samples sectioned on ultramicrotome (Leica, UC6) were stained in uranyl acetate and lead citrate than photographed on transmission electron microscope (FEI, Tecnai BioTWIN). In our

results it has been showed major changes on C6 cells ultrastructure indicating apoptosis caused by vanadyl sulphate as membrane blebbings, nuclear fragmentation, horseshoe nucleus and shrinkage on cells. From our findings it can be concluded that vanadyl sulphate possess anticarcinogenic effects on C6 cells showing its effects with clear apoptotic sparks and may be successful agent in drug desingning for cancer treatment but further investigations are required.

P307

Board Number: B442

Antitumoral effect of doxazosin on C6 rat glioma cells.

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Glioblastoma (GB) is the most frequent and most malignant human brain tumor. Prognosis for patients with GB remains dismal, as median survival after diagnosis varies from six months to one year. This is due to the inability of current treatment strategies to address the highly invasive nature of this disease. Thus, new therapeutic strategies are required. Doxazosin, a quinazoline compound, is a selective α 1-adrenoceptor antagonist used for treatment of high blood pressure and benign prostate tumor. In addition, it has also been demonstrated that doxazosin affects prostate cancer cells survival. In this context, the aim of this study was to evaluate the effect of doxazosin on C6 rat glioma cells. C6 cells were cultured with DMEM and treated with doxazosin at different concentrations. Cellular density and viability were determined by Suforhodamine B (SRB) and MTT assays, respectively. Cell death was analyzed by Propidium Iodide (PI) and Annexin V/PI staining. Cell cycle profiles were determined by flow cytometry (FACS Calibur, BD Bioscience). Cytotoxicity assays were performed in non-tumor cells, organotypic hippocampal culture slices and primary astrocyte cultures. Data are expressed as means \pm SD. All results are representative of at least 4 independent experiments. Differences between mean values were considered significant when $p < 0.05$. Doxazosin decreased cell density and induced cell death at concentrations that did not show toxic effect on non-tumor cells. Doxazosin also blocked cell cycle progression at the G1 phase at 150 μ M and 180 μ M, thus inhibiting cell proliferation. This study shows that doxazosin has antitumoral effects on C6 rat glioma cells at concentrations that are not toxic to non-tumor cells. This points to a promising perspective of further research on the effect of doxazosin for development of novel drugs for treatment of brain tumors.

P308**Board Number: B443****Discovery of a novel human pancreatic tumor stem cell targeting agent, CEP1430, through three-dimensional (3D) stem cell culture assay.**J.M. Harris¹, C.R. Sharma^{1,2}, M.R. Sharma¹, M.B. Navel¹, N. Amezcua¹, R.R. Rodriguez¹, S. Satish¹, J.R. Martinez¹, J. Jani¹, J.P. Sharma¹;¹Stem Cell Biology, Celprogen Inc, Torrance, CA, ²Celprogen Inc., Torrance, CA

Three-dimensional (3D) in vitro culture of cancer stem cells has long been advocated as a better model of the malignant phenotype that is most (more?) closely related to tumorigenicity in vivo. Moreover, new drug development requires simple in vitro models that resemble the in vivo situation to select most promising compound(s) utilizing minimal number of experimental animals.. Chemotherapy or concomitant chemoradiotherapy are frequently used in clinic to improve overall survival and organ preservation of cancer patients. Despite limited initial benefits, this approach encounters significant morbidity and mortality. Therefore reliable chemosensitivity assays are needed to accurately predict and guide clinicians to select the right individual treatment options for optimum benefit to cancer patients. . The purpose of this study is to examine and evaluate optimum drug candidates in vitro using primary patient tumor cells and cancer stem cells derived from tumors. . The primary pancreatic tumor samples obtained after surgery or biopsy, were placed immediately in Celprogen Tumor Transportation Media and shipped at 4-8 OC for processing. Tissues were washed with 1X PBS solution and aseptically cut into 0.5 mm sections and cultured in 6-well tissue culture plates with an insert pre-coated with ECM. All cancer cell types remain viable and maintain their native architecture for at least 14 days and incorporated DNA measured by adding EdU(5-ethynyl-2'-deoxyuridine) to the culture. In vitro efficacy of various therapeutic agents targeting major signaling pathways (wnt,Notch,PI3K,MAPK,STAT) and standard chemotherapeutic agents were tested using DNA uptake and TUNNEL assays to determine growth inhibition index. All test agents were also tested utilizing the patient's Pancreatic Cancer Stem Cell Cultures established with Celprogen's Media and ECM. Expression of PDX-1, SHH, CD24, CD44, CD133, EpCAM, CBX7, OCT4, SNAIL, SLUG, TWIST, Ki-67, E-cadherin, β -catenin and vimentin were quantified by qPCR or immunocytochemistry. Among the 1000 compounds screen tested, Gemcitabine, Taxol, Fluorouracil, Leucovorin, Irinotecan, and Oxaliptin were not effective against Pancreatic Cancer Stem cell (CSC) but were effective on tumor cells (differentiated CSCs). Our six lead small molecules [CEP1430,1431,1432,1433,1435&1436] were effective against Pancreatic CSC targeting selected pathways. CEP1430 shows promise as a better therapeutic agent against Pancreatic CSC and when tested in SCID mice model with once daily administration for 30 days without any adverse effects. CEP1430 reduced the tumor volume in the treated group by 80-90 %, when compared with the control group.

Cancer Stem Cells

P309

Board Number: B444

Profile of matrix metallo-proteinases (MMP) and chemokine receptors in prostate cancer stem cells.

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Background – Recently, we identified CD133+MDR1+Oct4+ prostate cancer stem cells (PCSCs) in human prostate cancer tissues. The matrix metallo-proteinase (MMP) profile and the expression of chemokine receptors of such progenitors is currently unknown and may affect their in vivo metastasis. In this study, we determined the matrix metallo-proteinase profile and the expression of chemokine receptors of prostate cancer progenitors. Methods and Results — PCSCs were isolated from prostate cancer tissue subjects and expression of matrix metallo-proteinase profile and the expression of chemokine receptors were determined. PCSCs had high expression of MMP-2, and MMP-9, moderate expression of MMP-7 and MMP11. Out of CXC chemokines receptors, CXCR4 is highly expressed and out of CC chemokines, CCR1, CCR2, CCR3 and CCR5 are highly expressed and other C chemokines and CX3C chemokines are minimally expressed. Moreover, PCSCs showed increased levels of SDF1 α and its binding to CXCR4 receptor. Conclusion — This data for the first time show a broad spectrum of matrix metallo-proteinase profile and the expression of chemokine receptors in prostate cancer progenitors and suggest the potential importance of matrix metallo-proteinase and chemokine receptors in mediating PCSCs mediated metastasis.

P310

Board Number: B445

Tetraspanin CD82 regulates acute myelogenous leukemia cell homing and β -catenin signaling through the stabilization and clustering of membrane-bound proteins.

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Patients with aggressive leukemia such as acute myelogenous leukemia (AML) often present with bone marrow failure because this form of leukemia homes and engrafts to the bone marrow, outcompeting normal hematopoiesis. In this aggressive form of AML, the surface expression of the membrane-bound tetraspanin, CD82, is often dysregulated. To understand how CD82 regulates the cellular behavior of AML, we used an AML cell line, KG1a, and stably generated CD82 knocked down (CD82KD) and overexpressing (CD82OE) cells. Interestingly when these cells were used for homing assays in NOD scid gamma mice, we found that CD82KD cells home to the bone marrow significantly less than control cells.

In parallel to these findings, primary AML patient samples with higher CD82 surface expression home significantly better to the bone marrow than patient samples with lower surface expression of CD82. We next wanted to assess the surface expression of adhesion molecules that play a role in homing to the bone marrow such as integrins, cadherins and selectins. We found that N-cadherin surface expression was significantly decreased on the CD82KD cells compared to control and CD82OE cells. Consistent with this finding, we observed a significant decrease in the binding of CD82KD cells to plate-bound N-cadherin when compared to CD82OE and control cells. We also observed that CD82 is immunoprecipitated with N-cadherin, signifying an interaction on the cell membrane and a point of potential cellular regulation. Next, we identified that β -catenin and down stream signaling were significantly dysregulated between all cells lines. We found that CD82KD cells have a marked increase in nuclear LEF1, a down stream target of active β -catenin signaling, where as the CD82OE cells had a significant decrease in LEF1 nuclear localization. Since clustering of N-cadherin can regulate β -catenin signaling, we wanted to determine if there was a CD82-mediated organizational difference of N-cadherin on the cell membrane. Using superresolution imaging, we found that N-cadherin and CD82 molecular clustering is significantly different between all cell lines. Additionally the glycosylation of CD82 has been implicated as a regulator of protein-protein interactions. We determined that cells overexpressing an unglycosylated form of CD82, home significantly better to the bone marrow than control cells and have a marked increase in both N-cadherin molecular density and CD82 cluster diameter. Together these data provide significant insight and the potential mechanism for how CD82 molecular organization dictates cellular behavior. Furthermore, these data highlight that while protein expression is important, cellular function is largely controlled by protein organization on the cell membrane.

P311

Board Number: B446

BLOCKADE OF ADENOSINE SIGNALING REVERSES CHEMORESISTANCE AND GROWTH OF GLIOBLASTOMA.

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Background. Glioblastoma multiforme (GBM) is the primary cause of death among brain cancer. Despite all efforts to improve the treatment currently, there is no cure for recurrence, mainly due to the phenomenon of multiple drug resistance (MDR) through MRP1/MRP3 transporters, which makes ineffective conventional chemotherapy. It has been attributed to the subpopulation Glioblastoma Stem-like Cells (GSCs) to be responsible of recurrence. Also, GSCs exhibit an extreme chemoresistance. Due to the fact that the nucleoside adenosine has recently been linked to the regulation of MRP transporters activity, our aim was to evaluate the blockade of both adenosine signaling or production on MDR in GSCs and experimental glioblastoma. **Methods.** Glioblastoma Stem-like Cells were derived from U87 glioblastoma cell line. The contents of Mrp1, Mrp3, adenosine receptors and 5'-ectonucleotidase were measured by western blots, flow cytometry and immunofluorescence. MRP1/MRP3 activity was

estimated measuring the extrusion of the fluorescent substrate CFDA from cells. Adenosine levels were quantified in Tyrode's buffer and 2-chloroacetaldehyde derivatives were quantified by HPLC. Cell viability was assayed by the MTT reduction method and flow cytometry. Apoptosis was assayed by flow cytometry. Cells were treated with the inhibitor of 5'-ectonucleotidase AOPCP, selective antagonists for adenosine receptors (DPCPX (30 nM), ZM241385 (10 nM), MRS1754 (50 nM), MRS1220 (10 μ M)) and the antitumoral drug vincristine (100nM). The effects on tumor growth were also evaluated in vivo. Results. GSCs were refractory to antitumoral drug vincristine. The antagonist of adenosine A3 receptor (MRS1220) and CD73 inhibitor (AOPCP) in combination with vincristine were the only treatments that showed a decrease in cell viability in vitro. Moreover, MRS1220-vincristine increased notably the percentage of apoptosis. In both adherents and GSCs, MRS1220 and AOPCP decreased the expression and activity of MRP1/MRP3. In vivo tests showed that co-therapy using MRS1220 decreases the tumor size by ~90% following administration for 9 days. In addition, this treatment caused a decreased expression of proliferation markers and MRP1/MRP3. Conclusions. Based on these findings we propose the use of adenosine A3 antagonists or 5'-ectonucleotidase inhibitors as coadjuvants for pro-apoptotic and chemosensitizing effects in GBM treatment. Supported by FONDECYT N°1121121

P312

Board Number: B447

The Effects of VEGFA in CD34+ cells.

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Angiogenesis plays a key role in growth and metastasis of solid tumors and recently has been reported in hematologic diseases. The angiogenic "switch" is mediated by several pro and anti-angiogenic factors and the most common is VEGFA. The VEGFA gene encodes the vascular endothelial growth factor which is a pro-angiogenic cytokine that can stimulate endothelial cells to proliferate, migrate and increases membrane permeability to plasma proteins. Several studies had described an increase of VEGFA expression in patients with acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) and this increase correlates to worse prognosis. Previous studies from our group found overexpression of VEGFA in CD34⁺ cells from patients with low-risk MDS in microarray assays. To validate these results, we selected 21 samples of CD34⁺ cells from bone marrow of 12 MDS patients (WHO classification: Low Risk: RARS= 03, RCMD= 03; and High Risk: RAEB I = 01, RAEB II= 05), 04 secondary AML patients, 05 *de novo* AML patients and 04 health donors. After analysis by Real-Time PCR, using HPRT as normalizer, we found a higher expression of VEGFA in low-risk MDS patients compared to control group and decrease of VEGFA expression during disease progression. We also found a statistically significant difference between the VEGFA expression in low-risk and *de novo* AML patients (2.95 [0.57-7.69], P=0.01Mann-Whitney test). To a better knowledge of VEGFA effects we isolated CD34⁺ cells from 03 umbilical cord blood and cultured then on DMEM medium (Dulbecco's Modified Eagle's Medium) supplemented with

20% fetal bovine serum, 50 µg/mL Stem Cell Factor, 30 µg/mL Interleukin 3 and VEGFA recombinant protein at concentrations of 25ng/mL, 50ng/mL or 100ng/mL for 96 hours. We performed apoptosis, cell cycle, cell proliferation and colony-forming assays and compared the results with CD34⁺ control cells (without VEGFA treatment). We identified a significant increase in cell proliferation ($118.2 \pm 2.7\%$ vs $100 \pm 0.5\%$, $P=0.02$ Paired t-test) and colony formation (111.9 ± 3.4 vs $88.88 \pm 5.1\%$, $P=0.01$ Paired t-test) in CD34⁺ cells treated with VEGFA 100 ng/mL compared to control cells. We found no statistical difference in apoptosis and cell cycle of CD34⁺ cells after VEGFA treatment. These results suggest that overexpression of VEGFA in CD34⁺ cells may confer advantage to the MDS stem cells of the patients, contributing to pathogenesis.

P313

Board Number: B448

Expression patterns of F-Actin and Nanog, an embryonic stem cell marker, in monolayer and spheroid culture of human liver carcinoma cell line.

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Recent studies report that cancer stem cells are responsible for tumor relapse, metastasis, and chemoresistance. Nanog, a cell-fate regulatory molecule known to be important for embryonic stem cell self-renewal, also plays a novel role in tumor development. Expression of Nanog in tumor tissue is correlated with the stage of disease and prognosis. It has also been suggested that heterogeneity in liver cancer is a result of the hierarchical organization of cancer stem cells. Actin is considered as another protein, which regulates stem cell fate decisions in tumour.

It was aimed to (i) examine expression patterns of F-actin and nanog in monolayer and spheroid culture of human liver hepatocellular carcinoma cell line (HepG2 cells), (ii) evaluate if the maintenance of 3D structure in culture effects presence and location of actin and nanog in cells and (iii) observe the changes at the level of these two proteins during different cultivation period.

HepG2 cells were seeded on coverslips. They were grown in Minimum Essential Medium (ATCC) which contain 10% fetal serum bovine. Cells, which were 60%, 80%, 100% confluent were fixed with acetone. After fixation, they were first labeled with fluorescent phallotoxins (Invitrogen). Same cells were also labeled with nanog(D73G4, Cell Signaling) as primary antibody and then anti-rabbit Dylight-550(Abcam). LSM700(Carl Zeiss) and Eclipse90i(Nikon) were used for examination of the expression of F-actin and nanog.

The results suggest that the actin cytoskeleton in monolayer cells show stress fiber formation while spheroids have cortical actin organization. When confluency increase, more peripherally located actin filaments become visible. The density of actin labeled regions per cell does not differ significantly while

the cultivation period increases. Labeling pattern and density are not correlated with the size of spheroids.

Cytoplasmic and nuclear staining for nanog is evident for some cells in different days during cultivation. The number of the cells labeled with nanog increases as the confluency increases. Some spheroids do not contain nanog-labeled cells while centrally located labeled cells are clear in the others. Staining pattern in monolayer cells and spheroids remain the same.

A number of studies have shown that tension forces and cell shape can determine cell fate in a variety of cell types. Actin distribution pattern in liver cancer cells might also be the part of this process. More studies are needed to clarify how stage and prognosis of the disease change according to the presence and different subcellular localization of nanog in cancer cells.

P314

Board Number: B449

Vascular niche specific bidirectional PDGF signaling mediated via glioma stem cell-endothelial cross-talk regulates tumor progression in glioblastoma.

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Glioblastoma (GBM) is a Grade IV glioma often displaying a highly vascular phenotype with extensive angiogenesis occurring due to a steadily proliferating endothelial cell (EC) population via paracrine interactions with glioma cells. Although, antiangiogenic therapies are emerging as front line treatment modality in glioblastoma, dearth of appropriate in vitro models to study glioma cell- endothelial cell interactions are essentially hindered due to lack of targetable novel signaling pathways. Human glioma stem cells (hGSC), surrounded by EC niche in tumor microenvironment, are known for tumor growth, therapeutic resistance, and recurrence. However, study of hGSC-EC physical interactions in cell culture system remains obscure till date due to unique growth conditions for each cell type. We have isolated multiple tumor matched hGSCs and ECs from primary human GBM tissues and developed novel 2D, 3D in vitro co culture systems to study role of tumor microenvironment and niche in glioma pathogenesis. Our in vivo studies demonstrate that mutual interactions of GSC-EC occur via niche specific PDGF signaling which promotes proliferation, migration of ECs thereby accelerating GBM angiogenesis. We using a 3D co-culture model of individually labeled GSCs and ECs show that GSCs extensively migrate on EC conduits while simultaneously promoting their proliferation. Next, we show that under radiation conditions hGSC migration is supported by endothelial cells and this could potentially be responsible for hGSCs escape from primary tumor during radiotherapy. Taken together, we show here that PDGF axis acts as a double edge sword for GBM which regulates angiogenesis and GSC migratory phenotype. Thus, targeting niche PDGF signaling cannot only curb GSC regulated angiogenesis, but also prevent EC assisted GSC migration. Additionally, we propose that our novel GSC-EC co-culture model has immense potential for in-depth studies to understand GSC-EC interactions and will provide an excellent platform to develop new therapeutics by identification of crucial niche pathways.

P315**Board Number: B450****Investigating the Role of Cancer Stem Cells in HeLa Cell Cultures.**D. Zhao¹, N.L. Marianelli²;¹Lycoming College, Williamsport, PA, ²Biology, Lycoming College, Williamsport, PA

The goal of our research is to study the role of cancer stem cells in the proliferation of HeLa cancer cells. Ultimately, we would like to develop a method for targeting cancer stem cells with chemotherapy as opposed to current methods which focus on only the cancer cells themselves, in hopes that without the presence of stem cells the cancer cells will undergo apoptosis. We stain for stem cell markers to confirm the presence of stem cells in the HeLa cell culture. We then remove these stem cells from the culture and conduct further research on their proliferation. We use fluorochoime-labeled antibodies and a fluorescent microscope to study the isolated stem cells. These stem cells undergo further experimentation, including Western Blotting, RT-PCR, and genome sequencing to determine levels of specific proteins and molecules within them. By this way we identify some molecules that can be targeted for treatment during chemotherapy.

P316**Board Number: B451****Characteristics of cancer stem cells in rhabdomyosarcoma.**D. Zhao¹, A.K. Follweiler²;¹Lycoming College, Williamsport, PA, ²Biology, Lycoming College, Williamsport, PA

Researchers have found that cancer cells are not all the same. Within a malignant tumor, there are a variety of types of cells. Cancer stem cells (CSCs) are cancer cells that have the ability to reproduce themselves and give rise to all cell types found in the tumors. Cancer stem cells (CSCs) have been identified in a number of tumors; however the CSCs of rhabdomyosarcoma (RMS) have not been identified. The goal of this study was to identify Stem Cells in RMS by side population analysis and characterize the population using the functional approach. We found that a particular population can be enriched from eRMS. This could a potential drug target for rhabdomyosarcoma (RMS).

P317**Board Number: B452****Identification of differentially expressed microRNAs defining heterogeneity of cancer cells by microarray chip analysis.**Y. Kim¹, M. Kim¹, N. Kim¹;¹Seoul National University, Seoul, Korea

Introduction/Objectives: Intratumoral heterogeneity is a histological hallmark in all tumor types and major barrier against effective cancer therapy. Especially, human malignant mesothelioma that is mainly associated with asbestos exposure is known to be extremely heterogeneous with regards to morphology as well as molecular phenotypes, resulting in poor response to conventional cancer therapy. This study was performed to investigate the global microRNA expression profile of distinct subpopulations of MS1 cell line, a human malignant mesothelioma cell line. **Materials and Methods:** The MS1 cells were cultured in RPMI1640 media with supplements and subjected to the side population (SP) assay composed of Hoechst 33342 dye staining and subsequent flow cytometry. Total RNAs were isolated from the sorted subpopulation cells, SP and non-SP fractions, followed by labeling and hybridization of miRNAs Affymetrix GeneChip miRNA Arrays for 16 hours at 48 °C and 60 rpm. After hybridization, the chips were stained and washed in a Genechip Fluidics Station 450 and scanned by using a Genechip Array scanner 3000 7G. After data extract and normalization, the analysis of differentially expressed microRNAs was performed. **Results:** A total of 294 miRNAs including 158 up-regulated and 136 down-regulated were identified based on the criteria of 1.5 fold difference and a p-value < 0.05, and a total of 16,983 target genes were identified. In ontology analysis, transcription regulator activity and positive regulation of gene expression were the most significantly enriched GO terms in biological process and molecular function categories, respectively.

Conclusion: The microarray for microRNA expression analysis revealed differentially regulated microRNAs between SP and NSP fractions.

Impact statement: This study will provide valuable information for the understanding of regulatory function of microRNAs in the generation and maintenance of more aggressive cancer cell subpopulations.

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Chromatin and Epigenetics

P318

Board Number: B454

Mitotic inhibition of the DNA double-strand break response preserves genomic integrity.

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Exogenous and endogenous DNA-damaging agents continuously threaten genome integrity and can cause cytotoxic DNA double-strand breaks (DSBs). Interphase cells have evolved dynamic and sensitive mechanisms to detect, signal, and repair DNA DSBs. A central component of this response is mediated through ubiquitin (Ub) signaling controlled by the E3 ubiquitin ligases RNF8/RNF168. RNF8/RNF168 ubiquitylate chromatin and promotes the recruitment of 53BP1 to DSBs, an important factor in classical non-homologous end-joining (C-NHEJ). In stark contrast, mitotic cells inactivate the Ub-dependent signaling and repair of DNA DSBs, a phenomenon first observed 60 years ago, but the significance of this inhibition remains elusive.

We investigated how mitosis blocks the DNA DSB response and determined the consequences of its reactivation. We identified a phosphorylation site in RNF8 downstream of its forkhead-associated (FHA) domain, which prevents the RNF8-MDC1 interaction and the initiation of the ubiquitylation of chromatin. Mutation of this site to alanine restores RNF8 recruitment to DSBs in mitosis, but fails to mobilize 53BP1 as monitored by immunofluorescence, suggesting the presence of a second inhibitory mechanism. We previously characterized an Ub-dependent recruitment (UDR) motif in 53BP1 that recognizes RNF168-dependent H2A Ub mark on nucleosomes. Interestingly, we observed that mitotic 53BP1 UDR motif is phosphorylated at two sites, which abrogates binding to ubiquitylated nucleosomes. Mutation of these two sites to alanine restores 53BP1 capacity to read ubiquitylated H2A and allows its recruitment to DSBs. Reactivation of RNF8 and 53BP1 in mitosis restores DNA repair, but is paradoxically deleterious. Indeed, restoration of the DNA DSB response in mitosis leads to Aurora B kinase-dependent sister chromatid fusions that produce dicentric chromosomes and the formation of micronuclei, a hallmark of aneuploidy.

Our findings reveal the complexity and the importance of regulating the DNA DSB response in mitosis. Through multiple phosphorylation events, mitotic cells block the Ub-dependent DNA DSB response, thus minimizing the risk of mitotic sister chromatid fusions.

P319**Board Number: B455****The Role of Centromere Defects in Cancer Formation and Progression.**T. Beh^{1,2}, B. Kim^{1,2}, P. Kalitsis^{1,2};¹Murdoch Children's Research Institute, Royal Children's Hospital, Parkville, Australia, ²Department of Paediatrics, The University of Melbourne, Parkville, Australia

The centromere is a unique region on each chromosome that functions as the site of kinetochore assembly and sister chromatid cohesion during mitosis and meiosis. Hence, the centromere plays a crucial role in genome inheritance, ensuring proper segregation of sister chromatids into each daughter cell. Despite its importance, the centromere has been relatively under investigated partly due to its reputation as part of the genomic 'junk DNA' post Human Genome Project. Its tract of highly repetitive sequences which spans several megabases also poses technical challenges for sequence assembly. Thus, to date, the centromeric sequence remains unresolved and its functional studies at the molecular level are inadequate. To contribute to the area of centromere research, my project aims to study centromere-related abnormalities using the NCI-60 panel comprising of 60 human cancer cell lines to characterize the spectrum of centromeric defects including occurrences of dicentric and neocentric chromosomes using the combination of fluorescent in situ hybridization (FISH) and immunocytochemistry. T-47D, a breast cancer cell line, was found to have high percentage of metaphase spreads with a neocentric chromosome which was identified via Multiplex-FISH to be a chromosome derived largely from Chromosome 3 with a small fragment of Chromosome 5. Chromatin immunoprecipitation (ChIP) using antibody against CENP-A followed by NextGen Sequencing revealed that the approximately 100kb neocentromere region was formed at the position 164,575,000 – 164,675,000bp adjacent to the SI (sucrase-isomaltase (alpha-glucosidase)) gene in the 3q26.1 band of Chromosome 3. This marks the first neocentromere reported in a human cancer cell line.

P320**Board Number: B456****Anti-SON protein "TSA-omics" Reveals a Gradient in Gene Density and Elevated Gene Expression as a Function of Proximity to Nuclear Speckles.**Y. Chen¹, Y. Zhang², J. Ma², A.S. Belmont¹;¹Department of Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, IL,²Department of Bioengineering, University of Illinois at Urbana-Champaign, Urbana, IL

Here we report development of a new genomic mapping method that measures genome proximity to nuclear speckles. Unexpectedly, this method also revealed Mbp-scale looping between the nuclear periphery and nuclear speckles from a single genomic plot. Genome-wide computational analysis demonstrates that a gradient in nuclear speckle proximity is mirrored by a gradient in both gene density and the distribution of highly expressed genes, with genomic regions closest to nuclear speckles showing peaks in gene density and the highest enrichment of highly expressed genes.

Our approach uses Tyramide Signal Amplification (TSA) immunostaining to create a gradient of biotin labeling from the target throughout the nucleus. Importantly, labeling can be directly visualized by immunofluorescence microscopy prior to DNA isolation and genomic analysis. Using nuclear speckle marker protein SON as the labeling target, we mapped the whole genome position relative to nuclear speckles in human erythroleukemia K562 cells, revealing distinct genome domains: speckle-proximal, speckle-distal, and connecting transition zones. Speckle-proximal domains correlate with gene-rich chromosome “R” bands, consistent with earlier cytological observations. Speckle-distal domains, appearing as valleys in the genomic proximity plot, correlate strongly with Lamina-Associated Domains (LADs). Transition zones extending over several Mbp frequently appear as linear ramps in the proximity plot, connecting valleys with peaks of speckle association. 3D immuno-FISH confirms that speckle-proximal domains associate at high frequency with nuclear speckles, while speckle-distal domains locate away from speckles, frequently near the nuclear periphery. FISH visualization of a 5Mbp transition zone revealed a linear signal typically stretching between the nuclear periphery and an interiorly located nuclear speckle, suggesting the capability of revealing Mbp-scale looping of interphase chromosomes between the nuclear periphery and nuclear speckles directly from the TSA genomic plot. Besides a gradient in gene density and highly expressed genes relative to nuclear speckle proximity, computational analysis also revealed a striking correlation of the density of specific histone modifications and chromatin-related marks with distance to nuclear speckles.

We anticipate this simple proximity-mapping method will provide a valuable tool to measure the spatial proximity of the genome to a variety of nuclear compartments. Simultaneous mapping of genome proximity to different nuclear compartments may allow deconvolution of 3D nuclear organization. The simplicity of this mapping approach makes it ideal for investigating the dynamics of nuclear organization and its correlation with changes in gene expression.

P321

Board Number: B457

Lambda exonuclease, the cornerstone of nascent strand sequencing to map replication origins genome-wide, digests GC-rich DNA inefficiently and stalls at G quadruplex structures.

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What defines metazoan origins of DNA replication remains poorly understood and suffers from a small sample size of well-characterized origins. Nascent strand sequencing (NS-seq) to discover origins genome-wide has the potential to vastly expand the set of known metazoan origins and thus identify features for their specification. Previous studies on individual origins concluded that origins are AT-rich. However, recent NS-seq results suggested that origins in multicellular eukaryotes are GC-rich and

correlated with G quadruplex (G4) structures. Therefore, G4s could be the long sought after feature that specifies metazoan origins. However, these conclusions rely upon the purity of the nascent strand DNA preparations in NS-seq that were enriched by Lambda exonuclease (Lexo), a 5' to 3' directed exonuclease that digests parental DNA while leaving nascent strands intact due to the presence of RNA primers at their 5' ends. Moreover, Bubble-Trap sequencing, an origin mapping technique that does not use Lexo, did not support the notion that G4s were a defining feature of metazoan origins. Thus, there is a need to understand the biases incurred by Lexo and whether Lexo enriches G4s independent of nascent strands.

We took biochemical and genomics approaches to determine if Lexo digests all parental DNA sequences equally. We report that Lexo does not efficiently digest G4 structures in a plasmid. Moreover, Lexo digestion of non-replicating genomic DNA (LexoG0) enriches GC-rich DNA and G4 structures genome-wide. We used LexoG0 data as a control to computationally eliminate potential false positives from NS-seq data and validated this approach at the rDNA locus. Whereas controlling with total genomic DNA results in enriching the entire rDNA locus, controlling with LexoG0 enriches only the known origins. When this LexoG0 control is applied genome-wide, NS-seq peaks are no longer GC-rich. In addition, only 6.8% of all G4s in the genome overlap with the corrected NS-seq peaks, suggesting that G4s are not a general determinant for origin specification. However, about a third of the NS-seq corrected peaks overlapped with G4s. In further analysis, we observed a periodic spacing of G4 motifs and nucleosomes around the peak summits suggesting that G4s may help to position nucleosomes. This may create a nucleosome-free region where the pre-replication complex can form at this subset of origins. Finally, we demonstrate that use of Na⁺ instead of K⁺ in the Lexo digestion buffer improves the ability of Lexo to digest G4s. Based on our computational and biochemical results, we propose ways to increase both the sensitivity and specificity of NS-seq for genome-wide identification of replication origins.

P322

Board Number: B458

Functional role of chromosome refolding during mating type switching in yeast.

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Haploid yeast, *Saccharomyces cerevisiae*, cells can switch their mating type by replacing one *MAT* allele with the DNA sequence of the opposite mating type, copied from a distant locus. Mating type switching in yeast is a highly choreographed repair event in which a DNA double strand break (DSB) created at the *MAT* locus is repaired by homologous recombination using one of the two silent donors, *HML* α or *HMR* α , located near the two ends on the same chromosome. *MAT* α cells recombine with *HML* 90% of the time, and this preference to use *HML* is dependent on a nearby cis-acting locus, the recombination enhancer (RE). The RE sequence physically interacts with the *MAT* locus after a DSB, thereby increasing the frequency of collisions between *HML* and *MAT*.

To observe the kinetics of switching and changes in chromosome conformation, we imaged two-color fluorescent reporters inserted near *MAT* and *HML*, and measured the three-dimensional distances between the two loci in live cells or in fixed cells that were collected at different time points before and after a DSB is induced. In addition we measured the percent usage of *HML* during *MAT* switching.

We have compared these experimental observations to a polymer model of yeast chromosome III to compute the distribution of distances between *MAT* and *HML* loci before and after the break. We find that the polymer model predictions are in quantitative agreement with the experimental distances before and after the break, thus establishing a biophysical basis of this gene conversion event. Moreover our calculations show that by refolding chromosome III after a DSB, via tethering RE at *MAT*, polymer entropy drives donor preference in yeast mating type switching.

P323

Board Number: B459

The organization and architecture of *Bacillus subtilis* chromosome revealed by super-resolution microscopy and HiC.

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In eukaryotes and bacteria, chromosome organization and segregation needs to be carefully orchestrated to ensure faithful transmission of genetic material during cell division. The molecular mechanisms responsible for bacterial chromosome organization and segregation remain elusive, possibly because these processes are highly influenced by the action of many DNA metabolic processes overlapping in time and space (e.g. replication, transcription, or repair). Here, we investigate the higher-order organization of DNA in *B. subtilis* by using a combination of chromosome-capture technologies and super-resolution microscopies. First, we found that specific barriers act to separate the chromosome into specific higher-order domains at different length-scales. Notably, higher-order domains are visible in single cells and their number increases with cell cycle progression. The number of domains and their genetic and cellular localization are found to depend on transcriptional activity. Secondly, our data reveal that replication severely affects the three-dimensional organization of the chromosome and highlight two molecular factors involved in this replication-induced re-organization. Finally, we determined the ultra-structural organization and dynamical behaviour of replication domains and their role in chromosome organization and segregation

P324**Board Number: B460****Labeling chromosomes in live cells: a CRISPR picture.**H. Ma¹, A. Nissen², P. ReyesGutierrez¹, S. Wolfe¹, S. Zhang², T. Pederson¹;¹Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA, ²Department of Electrical Engineering and Computer Science, University of Central Florida, Orlando, FL

Understanding the locations and dynamics of interphase chromosomes is a major goal in the field of nuclear organization and function. Fluorescence in situ hybridization (FISH) has been a major tool, manifest in its most all-encompassing form as Spectral Karyotyping (SKY), also known as. As important as these fixed cell methods are for a static picture, there is a parallel need to determine the locations and dynamics of interphase chromosomes in live cells, as has been done recently with TALEs. Here we report a multicolor CRISPR-based method for labeling individual chromosomes in human cells. It is based on computational mining of the human genome for chromosome-specific tandemly repetitive sequences combined with extensively optimized small guide RNAs for three different Cas9 orthologs. Using this method we have labeled the complete human karyotype. In confirmation of FISH results, we found in these live cell studies that the gene-rich chromosome 19 occupies a central position whereas the gene-poor chromosome 18 is located at the nuclear periphery. In addition, we found that chromosome 17 and the five acrocentric chromosomes (13, 14, 15, 21, 22) had central locations whereas chromosomes 3 and 7 tended to be located at or close to the nuclear periphery. We also examined the localization of the five Nucleolus Organizer Region-bearing acrocentric chromosomes in relation to nucleoli in diploid human RPE cells and observed that one copy of each chromosome was located close to a nucleolus, while the other was not. Finally, our method also labeled the heterochromatin at the pericentromeric region of 9q12. Most recently, we have developed dual-color versions of this method and are also applying it to study translocations in human tumor cells.

P325**Board Number: B461****Association of chromatin with the nuclear envelope supports stable nuclear mechanics.**S.M. Schreiner¹, P. Koo², Y. Zhao³, S. Mochrie³, M.C. King¹;¹Department of Cell Biology, Yale University, New Haven, CT, ²Physics, Yale University, New Haven, CT, ³Applied Physics, Yale University, New Haven, CT

Cells must constantly withstand mechanical stresses derived from both extracellular and intracellular forces. These forces can be transmitted to the cell's nucleus through connections with the cytoskeleton, raising the possibility that defects in nuclear mechanics can impact the cell's ability to withstand mechanical stress. The nuclear lamina, in particular lamin A, is often considered to be the primary mechanical defense of the mammalian nucleus. However, lamins are part of an integrated network of

proteins, lipids, and chromatin, all of which contribute to the ensemble mechanical properties of the nucleus. Yeast, which lack a nuclear lamina, provide a model system in which to study the lamin-independent contributions of chromatin and proteins residing in the nuclear membrane to nuclear mechanics. Here, we have combined a quantitative imaging platform capable of measuring 3D nuclear contours in live cells with an *in vitro* optical tweezers assay to probe the mechanical properties of *S. pombe* nuclei. In live cells, we find that association of chromatin with the inner nuclear membrane (INM) through integral membrane proteins is required for a normal mechanical response to microtubule (MT) forces. Increasing loss of integral INM proteins results in highly deformable nuclei that are subject to catastrophic failures in nuclear envelope integrity, specifically in response to exogenous forces from MTs. Loss of integral INM proteins that associate with the centromeres results in the most highly deformed nuclei *in vivo*. This observation supports a model in which the association of heterochromatin with the nuclear envelope adjacent to the spindle pole body, the yeast centrosome equivalent, helps buffer MT-driven forces. Further, our findings suggest that organisms without a nuclear lamina and with similar heterochromatin-centrosome interfaces have adopted a chromosome organization that contributes to the mechanical stability of nuclei. Using optical tweezers, we find that nuclei lacking integral INM proteins are less stiff than wild type nuclei, particularly when force is applied at rates that recapitulate the kinetics of MT dynamics *in vivo*. Wild type mitotic nuclei, in which chromatin is globally released from the INM, are extremely soft, raising the possibility that a transition in nuclear mechanics may support segregation of daughter nuclei during a period when forces are instead balanced between spindle poles. Together our data support a model in which the physical association of chromatin with the INM provides the stiffness necessary to buffer cytoskeletal forces in interphase while the deformability of mitotic nuclei supports nuclear division.

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Board Number: B462

Directed reorganization of chromatin to the nuclear lamina is mediated by chromatin state, YY1 and A-type lamins.

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Nuclear organization has been implicated in regulation of gene activity. Recently, large developmentally regulated regions of the genome that dynamically associate with the nuclear lamina, the so called Lamina Associated Domains (LADs), have been identified. Intriguingly the dynamic re-organization of these regions has been implicated in cell-type specific gene regulation. We and others have identified developmentally regulated genes that reside in these dynamically associating regions, which we call variable LAD (vLADs). However, little is known about the mechanisms underlying LAD organization, establishment and scaffolding to the nuclear lamina. In order to identify DNA sequences able to

establish a *de novo* LAD, we utilized our tagged chromosomal insertion site system (TCIS). Using TCIS we identified small sequences from borders of fibroblast specific vLADs that are sufficient to target these ectopic sites to the nuclear periphery. These relocating sequences are enriched in motifs for Ying-Yang1 (YY1), BTB/POZ domain transcription factors (i.e. Zbtb7b) and CTCF. Knockdown of these proteins or lamin A/C, but not lamin A, led to a loss of lamina association. In addition, targeted recruitment of YY1 proteins facilitated ectopic LAD formation dependent on histone H3 lysine 27 trimethylation (H3K27me3) and histone H3 lysine di- and tri-methylation (H3K9me2/3). Additionally, we show that endogenous loci are dependent upon lamin A/C, YY1, H3K27me3 and H3K9me2/3 for maintenance of lamina proximal positioning. Taken together, these results reveal a mechanism for LAD recruitment implicating the involvement of tissue specific factors and epigenetic modifications.

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Board Number: B463

Repo-Man is a repressive chromatin modulator in the interphase nuclei.

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Upon mitotic exit, the establishment of the nuclear lamina on the newly formed G1 nucleus coupled to a controlled program of chromosome de-condensation ensures the organisation of chromatin inside the nucleus. The transition from mitosis to interphase requires the removal of mitotic histone modifications; Repo-Man/PP1 is one of the phosphatases involved in this process and specifically removes phospho-residues on the H3 tail (T3, S10, and S28). In the G1 nuclei, Repo-Man is nuclear with enrichment at the nuclear periphery. The nuclear periphery is associated with transcription repression due to the absence of transcription machinery (e.g. RNAPII) and accumulation of repressive chromatin modifiers and histone marks. Here we show that Repo-Man is important for chromatin organisation through association with components of the Nuclear Pore Complex (NPC) and formation of a repressive heterochromatic environment. Repo-Man is tethered to the nuclear periphery via the nucleoporin Nup153 that we had previously identified in a proteomic screen for Repo-Man interactors. Repo-Man establishes interactions with chromatin at the nuclear periphery possibly via its Histone binding domain, as its depletion causes the re-localisation of the peripheral bound Chr13q towards the nuclear interior. Finally, targeting of Repo-Man to a specific locus is sufficient to generate local enrichment of repressive marks of transcription, such as H3K27me3 and HP1. And, consistent with this, depletion of Repo-Man compromises the distribution of HP1 foci. These data suggest that Repo-Man is involved in shaping chromatin organisation and forming nuclear architecture upon mitotic exit.

P328**Board Number: B464****Principles of Self-Organizing Genome Architecture During Human Muscle Cell Differentiation.**D. Neems¹, S.T. Kosak¹, A. Garza-Gongora¹;¹Department of Cell and Molecular Biology, Northwestern University, Feinberg School of Medicine, Chicago, IL

The organization of the human genome within the nucleus is non-random; however, the guiding principles of this behavior are not fully understood. Our work suggests that the three-dimensional configuration of the genome is related to a linear distribution of co-regulated genes. Using myogenesis as a model system, we seek to uncover how these linear gene clusters may give rise to lineage specific nuclear topologies. Myogenesis represents an ideal system for studying this phenomenon, as it is easily amenable to modeling and the formation of syncytia provides unique experimental opportunities, such as studying the effects of the cell cycle. Using whole genome expression analysis during the process of myogenesis, we have identified human chromosome 1 (HSA1) to be a 'hotspot' for linear clusters of differentially regulated muscle-specific genes. We have found both long linear stretches of contiguous co-regulated genes and a significant number of topologically associated domains (TADs) enriched for myogenic genes. Importantly, we find that myogenin, a master regulator of muscle development, is within a TAD enriched for other myogenic genes. This TAD undergoes a significant change in nuclear organization upon differentiation, whereas other highly expressed genes found within HSA1 but outside enriched TADs do not. The myogenin-containing TAD's pattern of organization results in repositioning away from the nuclear periphery in conjunction with a reduction in inter-allelic distance. To determine if this developmental reorganization is specific, we simultaneously examined the myogenin-containing TAD and those upstream and downstream, creating three unique signals over a 1 Mbp region. The myogenin TAD showed a more dramatic phenotype than either neighboring region, confirming that activity was derived from that domain. Furthermore, the single cell nature of our study allowed us to confirm a high propensity for myogenin allele polarization with regards to inter-HSA1 distance. The observed reduced inter-allelic distance may serve a function in stabilizing the expression from each allele by bringing them into the same sub-nuclear microenvironment. This conclusion is supported by RNA-FISH experiments that show a positive correlation between intensity differences and microns between RNA-FISH signals in myotubes with two unique signals. Finally, taking advantage of naturally occurring cell fusion in myogenesis, we are also able to determine the cell cycle dependency of the various phenomenon observed during syncytial formation. We propose that the linear gene distribution, specifically the co-linearity of developmentally regulated genes, underlie observed changes in three-dimensional positioning and may indicate epigenetic regulation through genome organization.

P329**Board Number: B465****Analysis of foci created by the telomere binding protein POT-1 in *C. elegans*.**F. Peng¹, L. Shtessel², S. Ahmed^{2,3}, P.S. Maddox³;¹Curriculum in Genetics and Molecular Biology, University of North Carolina, Chapel Hill, NC,²Department of Genetics, University of North Carolina, Chapel Hill, NC, ³Department of Biology, University of North Carolina, Chapel Hill, NC

Telomeres are tracts of tandem DNA repeats that protect the ends of linear chromosomes from replicative erosion and from being identified and repaired as double-strand breaks. Shelterin is a complex of proteins found to directly associate with telomeres and functions to distinguish telomeres from double-strand breaks. The shelterin component POT1 binds to single stranded telomeric DNA, which is present at the chromosome terminus and also likely at the displacement loop (T-loop) created by strand invasion of the 3' overhang into the telomeric duplex. POT1 homologues have recently been identified and characterised in *C. elegans*. A transgene expressing *pot-1::mCherry* reveals strong punctate foci within the germline corresponding to discrete telomeric termini. Here, we examine the dynamics of POT-1 foci in the germline and during early development within living embryos using time-lapse imaging. Using various end-to-end chromosomal fusions, we interrogate the behaviour of POT-1 in the context of fused chromosomal ends harbouring telomeric repeats of varying lengths and show that creation of POT-1 foci does not require a chromosome terminus. We also analyse POT-1::mCherry foci in genetic backgrounds where telomerase activity is abolished, leading to shortened telomeres, and in mutants causing telomere elongation, as occurs with mutant *pot-1*. Our results suggest that POT-1 creates large, discrete structures within the nucleus that, perhaps surprisingly, are independent of both telomere length and of chromosome termini. We are testing the hypothesis that dynamics of POT-1 foci during development could be relevant to telomere biology.

P330**Board Number: B466****Annotation of genes located on the highly heterochromatic F element in *Drosophila biarmipes*.**A.R. Kneubehl¹, L.E. Guerriero¹, R.E. Boody¹, J.L. Sanford¹;¹Biological and Allied Health Sciences, Ohio Northern University, Ada, OH

The Muller F element, chromosome four in *Drosophila*, is commonly referred to as the dot chromosome and is unique among *Drosophila* autosomes in that it is highly heterochromatic and contains a high density of repetitive sequence. Furthermore, comparative genomic analyses of the F element shows that it contains a similar proportion of active genes as compared to euchromatic control regions. The Genomics Education Partnership (GEP) of Washington University, St. Louis strives to elucidate the evolutionary and gene regulatory mechanics behind this unique gene expression. Undergraduate researchers from colleges and universities across the nation perform sequence improvement and

manually annotate genes of recently sequenced *Drosophila* species. The present study focuses on annotation of contigs 3, 22, 37, 42, and 43 of the *Drosophila biarmipes* assembly, using standard annotation protocols. Briefly, NCBI BLAST was utilized for sequence alignment of contigs with the *Drosophila melanogaster* reference sequence and the UCSC Genome Browser was used to create gene models of all gene isoforms located on the investigated contigs. Results indicated that contig 3 of the *D. biarmipes* assembly contains the Mes2 gene, while contig 22 contains the CG6914 and CG14459 genes, and contig 37 contains the Oct-TyrR and CG7139 genes. Annotation of contig 42 and 43 revealed the presence of the bent gene on both contigs. Contig 42 contains the entirety of the bent gene; whereas, contig 43 contains half of the bent gene and all exons of the MED26 gene. The final gene models constructed from the annotation of these genes include: start and stop coordinates, exon coordinates for all the gene isoforms and verification of canonical splice donor and splice acceptor sites. Finally, dot plot analysis revealed sequence conservation between the *D. biarmipes* genes and their *D. melanogaster* orthologue. Future work will focus on annotating the transcription start sites for these genes, as well motif hunting to discover conserved sequence motifs that may be involved in regulating the expression of these F element genes. Overall, analyses of the gene structure of F element genes across multiple *Drosophila* species, including genes annotated in this study, will help to elucidate the cellular mechanisms responsible for gene expression within highly heterochromatic regions.

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Board Number: B467

Basal activity of a PARP1-NuA4 complex varies dramatically between breast cancer cell lines.

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Poly(ADP-ribose) (PAR) is an important but poorly understood post-translational modification involved in multiple biological processes including transcription, DNA damage repair and other stress responses. Control of protein PARylation is a balance between the activity of multiple poly(ADP-ribose) polymerases (PARPs) that catalyze the serial addition of ADP-ribose onto acceptor proteins to form linear and branched chains, and a PAR glycohydrolase (PARG) removing this modification from the protein surface. Despite their importance, fundamental questions about the regulation and cellular functions of PARPs remain unanswered. We are addressing these questions through an improved quantitative assay measuring PARP activity in cell lysates and through biochemical and proteomics methods determining PARP interaction partners. Much of the work done on PARPs has focused on their role in DNA damage. To gain insight into other potentially critical roles, we took an unbiased approach and analyzed cell lines grown under normal conditions in the absence of DNA damage. We found that basal PARP1 activity varies across breast cancer cell lines. The increase in basal activity appears to be independent of DNA damage, the most characterized PARP1 activation mechanism. The observed increase in PARP1 activity in some cell lines is due to a small fraction of activated PARP1. The activated

PARP1 is in complex with the chromatin remodeling complex NuA4, and the activity of PARP1 is dependent upon subunits of NuA4. Our findings demonstrate that PARP1 exists in multiple distinct activation states both within cells and between cell types, and we identified a new interaction/activation partner for PARP1. Overall, these studies expand our basic biochemical understanding of PARP1 and our understanding of its role in aspects of cell physiology outside of the DNA damage response.

P332

Board Number: B468

Glucose causes neurodegeneration in induced orexin neurons.

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Degeneration of orexin-producing neurons causes diseases related to sleep, metabolism, and motivation, and it is considered to be caused by physiological changes such as aging or the onset of diabetes. Analyses on orexin neurons are difficult since orexin neurons exist only thousands of cells in hypothalamus. We previously reported that mouse embryonic stem cells could be induced to differentiate into orexin neurons by treating them with N-acetyl-D-mannosamine (ManNAc). This induction is achieved by the repression of O-GlcNAc transferase (Ogt) and the histone de-acetylase Sirt1 on the *Hcr* gene locus coding for prepro-orexin. Therefore, this culture system can be utilized to explore the factors and mechanisms of neurodegeneration in orexin neurons. Here, we aimed to generate orexin neurons from human induced pluripotent stem cells (hiPSCs) and to find the cause of neurodegeneration. Twenty days after the neural induction, we detected *HCRT* gene expression in the cells cultured in a medium supplemented with ManNAc. The hiPSCs-derived *HCRT*-expressing cells behaved similar to the *in vivo* cells, because orexin peptides were released to the medium owing to the treatment with ghrelin, which was in contrast to the result obtained following treatment with leptin, Npy, and glucose. In these induced orexin neurons, the *HCRT* gene showed higher acetylation on H3/H4. Concomitantly, histone acetyltransferase, p300, CBP, and MEGA5 (also called O-GlcNAcase) were colocalized at the *HCRT* gene locus in the orexin neurons. Considering the fact that treatment with OGT and SIRT1 inhibitors could increase the induction efficiency of orexin neurons from hiPSCs to approximately five-fold, the suppression of these proteins was a critical epigenetic event in orexin neurogenesis, common to humans and mice alike. To study the mechanism of loss of orexin neurons, we evaluated orexin expression in long-term culture. When orexin neurons derived from hiPSCs were cultured for over 20 days, we observed a dramatic reduction in the *HCRT* expression. Generally, in the culture medium for neural cells, glucose is present at high concentrations as compared to its concentration *in vivo*. Accordingly the *in vivo*, glucose concentration was reduced to 5 mM during long-term culture, and the results indicated that the *HCRT* expression was maintained in associated with H3/H4 hyper-acetylation and hypo-O-GlcNAcylation on the *HCRT* locus. In summary, functional orexin neurons were successfully induced from hiPSCs after increasing histone acetylation and suppression of O-GlcNAcylation and using this culture system, we revealed that glucose was involved in the degeneration of orexin neurons.

P333**Board Number: B469****Linker histone variant H1T targets rDNA repeats.**R. Tani¹, K. Hayakawa¹, S. . Tanaka¹, K. Shiota¹;¹Dep. of Anim. Res. Sci., The University of Tokyo, Tokyo, Japan

Linker histone H1 is composed of a short N-terminal domain, a central globular domain, and a long C-terminal domain (CTD). Germinal-cell-specific variants exhibit low homology with the somatic variants, particularly in the CTD. Based on the studies of somatic H1 variants, H1 is thought to condense the chromatin structures, leading to a repression of gene expression. However, our recent study revealed that H1foo, a germinal-cell-specific variant, decondensed the chromatin structures in specific regions. H1T is also a germinal-cell-specific variant and is expressed from the primary spermatocyte stage to the early spermatid stage during spermatogenesis. We hypothesized that germinal-cell-specific H1 variants possessed different functions from those of the somatic variants and had unique targeting loci. In this study, we found that the expression of H1T was detectable in tumor cell lines such as AGS and MDA-MB-231, although the expression levels were much lower than those found in the testis. Additionally, H1T was also expressed in multipotent stem cells such as mouse embryonic stem cells (mESCs), and even in myoblast cells and gastric cells. Fluorescence immunostaining revealed that H1T and the somatic H1 variants were localized in the nuclei. Interestingly, H1T was accumulated in the nucleoli regardless of cell type, whereas H1C, a somatic variant, was accumulated only in some of the cell lines.

To clarify the function of H1T, we identified H1T target loci in AGS, MDA-MB-231, and mESCs using ChIP-seq analyses. H1T, unlike the somatic H1 variants, was found to be enriched particularly in the clusters of repeated rRNA genes (rDNA repeats) in all the analyzed cells. The distribution of H1T in the rDNA region was validated through ChIP-QPCR and was compared with the H1C distribution to obtain details of their respective distribution patterns in the rDNA. H1T was enriched mostly around the transcription start sites of the pre-rRNA. To reveal the function of H1T to rDNA repeats, knockdown and overexpression experiments revealed that H1T functioned as a repressor of rDNA transcription.

These data, together with our previous report about H1foo, support the hypothesis that germinal-cell-specific H1 variants have specific biological functions. In conclusion, H1T uniquely targets rDNA repeats to repress transcription.

P334**Board Number: B470****A new paternal effect lethal is required to prime paternal chromatin for embryonic mitosis in *Drosophila*.**M.T. Levine¹, H.M. van der Wende¹, H.S. Malik²;¹Fred Hutchinson Cancer Research Center, Seattle, WA, ²HHMI and Fred Hutchinson Cancer Research Center, Seattle, WA

Eukaryotic DNA is packaged into distinct chromatin compartments that support essential, highly conserved functions. Many chromatin proteins, however, are unconserved. To gain insight into this paradox, we have characterized the ancient Heterochromatin Protein 1E (HP1E) gene in *Drosophila*, which is essential for male fertility in *D. melanogaster* but recurrently lost over evolutionary time. We report here that HP1E is a new paternal effect lethal (PEL). HP1E-depleted fathers make motile sperm that deposit paternal DNA into the egg, but these embryos arrest after only a few rounds of mitotic division. During the 1st zygotic mitosis, we observe an anaphase bridge comprised of paternal chromatin. We could rescue mitotic cycling by crossing these PEL fathers to sesame- mutant mothers, whose embryos bypass paternal DNA and instead undergo maternal haploid mitosis. This mitotic rescue implicates the paternal chromatin as the primary source of the PEL-induced embryonic arrest. Consistent with this inference, HP1E localizes to wildtype post-meiotic spermatid nuclei and persists through sperm development but disappears at sperm maturation. Our data suggest that HP1E must act pre-fertilization—during spermiogenesis—to prime the paternal genome for embryonic chromosome segregation post-fertilization. HP1E's essential function in embryonic mitosis in *D. melanogaster* makes its repeated pseudogenization in other species puzzling. In the obscura group of *Drosophila*, however, HP1E loss coincides precisely with a major karyotype rearrangement involving the highly heterochromatic X- and Y- chromosomes, including the birth of a neo-Y. This phylogenetic correlation raised the possibility of a synthetic interaction between HP1E and the sex chromosomes. To test this prediction in *D. melanogaster*, we conducted chromosome-specific, fluorescent in situ hybridization on PEL embryos fathered by HP1E-depleted males. We discovered significant enrichment only of the paternal X- and Y- chromosomes in the telophase bridges of female and male embryos, respectively. The autosomes, in contrast, segregated faithfully. The sex chromosomes harbor the longest tracts of heterochromatin across the *D. melanogaster* karyotype. We propose that HP1E depletion in the male germline results in incomplete heterochromatic DNA replication prior to the first embryonic mitosis. Under this model, a shift in the euchromatin-heterochromatin boundaries upon sex chromosome rearrangement in the obscura group released functional constraint on a once-essential HP1 gene.

P335**Board Number: B471****Effective and novel nuclear RIP protocols to study interactions between proteins and non-coding RNAs.**M.H. Hsu¹, K. Saito¹, J. Rosenfeld¹;¹Cell Biology, EMD Millipore, Temecula, CA

Chromatin is typically thought of as a complex of DNA, histones, and non-histone proteins. The RNA component of chromatin was considered to be mRNAs or traditional snRNAs that would transiently associate with chromatin during transcription. However, mounting evidence suggests that various classes of non-coding RNAs are associated with chromatin and serve regulatory functions possibly through sequence-specific hybridization and/or through structural and spatial mechanisms. Approaches that allow one to identify and characterize interactions between RNA molecules (both coding and non-coding), proteins and DNA are needed to better characterize these regulatory mechanisms.

One approach used to detect and identify non-coding RNA molecules bound to regulatory complexes is RNA-binding protein immunoprecipitation (RIP). This method allows the immunoprecipitation of protein:RNA complexes. However it is not specifically designed for the study of chromatin associated RNAs. To enable the use of RIP to study RNAs associated with chromatin, we have developed optimized methods to isolate and detect protein-associated long non-coding RNAs (lncRNAs) in chromatin. This approach is designed to allow the discovery and analysis of non-coding RNA regulatory functions. We have developed protocols for using either cross-linked or non-cross linked (native) starting materials thus allowing different types of interactions to be detected (e.g. direct vs. indirect). Our protocols show improved signal-to-noise ratios, work with varied amounts of starting materials and enable downstream analysis by either qRT-PCR or Next-Generation Sequencing. Here we present results showing the interaction of various long non-coding RNA molecules with protein complexes.

P336**Board Number: B472****Role for centromeric epigenetic regulation post terminal, non-proliferative, differentiation.**L.I. Smith¹, P.S. Maddox²;¹Genetics and Molecular Biology, University of North Carolina, Chapel Hill, NC, ²Department of Biology, University of North Carolina, Chapel Hill, NC

Centromeres are large genomic loci that act as the scaffolding point for kinetochore assembly and are necessary for microtubule based chromosome segregation during cell division. CENP-A is a histone H3 variant that serves as the epigenetic mark of centromeres and is critical for maintaining genomic stability. Although the role of centromeres in cell division is relatively well studied, very little has been done to explore the role of centromeres once a cell has terminally differentiated. We reasoned that

such a large genomic body might have a non-proliferative role in cell homeostasis. To determine if centromeres are required in terminally differentiated cells, we disrupted centromeres in adult *C. elegans* which consist of ~1,000 terminally differentiated, non-proliferative, cells and only a few stem cells (located in the germline and not required for viability). We used RNAi based protein depletion to knock down KNL-2, the most upstream regulator of CENP-A maintenance in chromosomes. In early *C. elegans* embryos, loss of KNL-2 function leads to failure of CENP-A to localize to centromeric regions. We hypothesized that KNL-2 depletion in adult animals would lead to loss of centromeric identity and reveal any hidden role for centromeres in non-proliferative cells. Adult wild type worms depleted of KNL-2 were found to have significantly shortened lifespans, indicating a possible dependency for centromeric integrity post-differentiation. We repeated the experiment in adult worms that completely lacked a germline (stem cells or somatic tissue), and found an even more significant reduction in lifespan in these worms. The CENP-A protein itself is quite long lived as revealed in pulse chase experiments in human tissue culture cells. We found that RNAi depletion of CENP-A itself did not affect lifespan in a manner significantly different from depleting a mitosis specific kinetochore protein (HIM-10) in wild type worms, likely due to the long-lived nature of CENP-A protein. However, depletion of CENP-A did significantly decrease lifespan in the longer lived gonadless worms, likely due to the increased lifespan allowing the long lived CENP-A embedded in chromatin to eventually be depleted. Our data suggest that centromere maintenance by KNL-2, is important for maintaining a healthy organism, although we can not rule out a non-centromere role for KNL-2 in post-mitotic cells. Importantly, the shortening of lifespan observed is independent of any abnormal stem cell divisions. In sum, this analysis supports the idea that the structural integrity of centromeres does have an important role post differentiation and cannot simply be discarded as a structural remnant of cell division.

P337

Board Number: B473

A Role for Nuclear Actin in Regulating HDAC 1 and 2 Activity.

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Class I histone deacetylases (HDACs) are known to remove acetyl groups from histone tails. This liberates positive charges on the histone tail and allows for tighter winding of DNA, preventing transcription factor interaction and gene activation. Although the functions of HDAC proteins are becoming apparent both biochemically and clinically, how this class of proteins is regulated remains poorly understood. Pulldown screens have identified a novel interaction between nuclear actin and HDAC1 and HDAC 2. Nuclear actin has been previously shown to interact with a growing host of nuclear proteins including chromatin remodeling complexes, transcription factors, and RNA polymerases. We find the interaction between Class I HDACs and nuclear actin is activity dependent as treatment with an HDAC inhibitor, Trichostatin A, decreases their association. ChIP assays on activated and Trichostatin A treated cells show an inverse correlation between HDAC and nuclear actin binding of DNA. Furthermore,

we find that increasing the concentration of actin in HeLa nuclear extracts is able to suppress overall HDAC function as measured by substrate deacetylation. Together our data identify a novel mechanism by which nuclear actin is able to bind the active HDAC 1 and 2 complexes and inhibit deacetylase activities.

P338

Board Number: B474

Non-random arrangement of chromosomes 1, 29 and X in bovine spermatozoa.

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Comprehensive studies of chromatin organization in human spermatozoa have demonstrated that, similarly to somatic cells, chromosomes of male gametes are organized in distinctive territories that are non-randomly located in the nucleus (Zalensky et al., 1993, 1995; Zalenskaya, Zalensky 2004, 2007; Mudrak et al., 2005; Manvelyan et al., 2008; Mudrak et al., 2012).

Apparently, these features have been preserved throughout the evolution of mammals as evidenced from studies of chromosome localization in spermatozoa of monotremes and marsupials (Greaves et al., 2001; Greaves et al., 2003), rats (Meyer-Ficca et al., 1998), and pigs (Foster et al., 2005).

The arrangement of chromosomes in the sperm nucleus may have a functional significance by influencing the sequence of post-fertilization events.

The chromosome arrangement in spermatozoa of livestock species was studied only in pigs (Foster et al., 2005). Studies of bovine male gametes were limited to identification of sex chromosomes for the selection of X and Y spermatozoa (Sharpe, Evans, 2009).

Here, using fluorescence in situ hybridization (FISH) with whole chromosome painting probes and a recently developed statistical approach (Mudrak et al., 2012), we investigate intranuclear locations of chromosomes 1 (CHR 1) and 29 (CHR 29), the largest and the smallest autosomes within the bovine karyotype, and chromosome X (CHR X) in mature spermatozoa of *Bos taurus* (domestic bulls).

We show that all these chromosomes demonstrate the preferred lateral and longitudinal intranuclear distributions in the elongated sperm nuclei. CHR1 and CHR 29 tend to be located at the opposite ends of the sperm nuclei in the longitudinal direction: CHR 1 is preferentially found in the acrosomal part, and CHR 29 in the basal part of the nucleus. Laterally, CHR 29 is located towards the nuclear periphery as compared with the more internal position of CHR 1. CHR X is located in the medial third of the nucleus, shifted towards the apical third (on the border between the apical and medial thirds) longitudinally, and is located in the center of the nucleus laterally.

We demonstrate that the patterns of intranuclear location of chromosomes in bovine spermatozoa are non-random and specific for individual chromosomes, suggesting the existence of chromatin positioning factors. Studies of the chromatin remodeling events at early stages of embryogenesis will be essential

for understanding the functional aspects of non-random genome architecture in spermatozoa.

P339

Board Number: B475

Chromosome end protection and telomere length maintenance *cdc13-1* strains.

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Telomeres, the physical ends of eukaryotic chromosomes, function to protect DNA ends from degradation and from end to end fusion. Telomeres consist of stretches of repeated C/G-rich DNA ending with 3' single stranded G-rich overhangs. Telomere maintenance and function are facilitated by the enzyme telomerase and by many accessory proteins. Cdc13p is an essential, G-strand binding protein that functions in telomere protection and in telomerase recruitment. *cdc13-1* is a temperature sensitive allele of *CDC13*, that is defective for telomere end protection and at elevated temperatures, *cdc13-1* strains exhibit extensive C-strand strand specific degradation and show a G2/M cell cycle arrest. However, at permissive temperatures for growth, telomere length and end protection are similar to wild-type. Ku is a non-essential heterodimer composed of Ku70p and Ku80p. Ku plays multiple roles in DNA metabolism including: non-homologous end joining, recombination, telomerase recruitment and telomere end protection. The capping function of γ KU is necessary in G1. *cdc13-1*, *yku* Δ double mutants strains exhibit enhanced temperature sensitivity and impaired growth. This study examines the effect of a panel of mutations in γ KU80 on *cdc13-1* strains. Cells were monitored for growth at permissive and semi-permissive temperatures and telomere length were assayed by Southern blot. We also examined genetic interactions between *cdc13-1* and *yku80* mutant alleles and Pif1p, a helicase that inhibits telomerase activity. Our results suggest that Pif1p activity helps to promote normal telomeres length at permissive temperatures in *cdc13-1* strains, perhaps by removing telomerase that is telomere associated via an interaction with γ KU.

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Board Number: B476

Construction of Human Artificial Chromosomes in a non-transformed cell line.

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Human Artificial Chromosomes (HACs) are extrachromosomal elements that contain a functional centromere and behave as normal chromosomes. HACs have been used to study the epigenetic regulation of centromeres and they have also been proposed as an alternative gene-delivery system

since they have a number of advantages over the current viral-based methods. However, a significant limitation of this technology is that only the human HT1080 cell line is routinely competent for de novo HAC formation. HT1080 is a transformed cell line that shows chromosome instability (CIN). The aim of this project was to construct a new HAC in a non-transformed cell line (non-CIN) in order to obtain a better tool for future chromosome biology studies. We generated a synthetic alphoid-DNA array based on the type-I satellite DNA from chromosome 21 by RCA-TAR cloning (21alphoidtetO array). This synthetic array contains tetracycline operators in every second monomer replacing the 17-bp CENP-B-box sequence. The 21alphoidtetO array was transfected into the non-transformed cell line RPE1-hTERT (46, XX; der(X)) transiently overexpressing either Mis18 α , HJURP or CENP-28 (factors involved in CENP-A recruitment) as tetR-fusion proteins. RPE1-hTERT cells overexpressing tetR-EYFP protein were used as a control. Transfectants were selected with geneticin for the vector containing the 21alphoidtetO array for approximately 21 days. Individual clones were further expanded for 7 days in the presence of geneticin. Metaphase-chromosome spreads were prepared for Fluorescence in situ Hybridization (FISH) using a DNA probe for alpha-satellite21 labelled with Spectrum Green (Abbott Molecular, Inc). A minimum of 50 metaphases were analysed in order to assess the frequency of HAC formation in each condition. Importantly, our experiments detected HAC formation in the RPE1-hTERT cells that were transfected with the 21alphoidtetO array while also transiently overexpressing Mis18 α , HJURP or CENP-28 tetR-fusion proteins. The frequency of HAC-containing metaphases in these clones was different when Mis18 α , HJURP and CENP-28 tetR-fusion proteins were overexpressed (6.55%, 4.1% and 2%, respectively). As expected, no HAC-containing metaphases were observed when the 21alphoidtetO array was transfected in cells transiently overexpressing the tetR protein as a control. Our results suggest that Mis18 α may license the chromatin for de novo kinetochore formation more efficiently than HJURP or CENP-28. Furthermore, these data showed the possibility of constructing HACs in cell lines other than HT1080. Therefore, this approach represents an important advance in HAC technology both for future chromosome biology studies and for their potential use as gene-delivery vectors.

Nuclear Lamina and Laminopathies

P341

Board Number: B478

Abnormal processing of prelamin A in progeria affects nuclear movement and cell migration.

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Hutchinson-Gilford progeria syndrome (HGPS) is a rare genetic disease characterized by appearance of aging phenotypes in childhood. HGPS is caused by mutations in the LMNA gene that generate a truncated prelamin A (progerin) that cannot be processed and remains farnesylated. Lamin A anchors LINC complex nesprin and SUN proteins and we found by fluorescence recovery after photobleaching

that progerin expression reduced the mobility of nesprin-1, nesprin-2, and SUN2, but not that of nesprin-3, nesprin-4 or SUN1. Actin-dependent nuclear movement in NIH3T3 fibroblasts polarizing for migration requires the formation of nesprin-2G and SUN2 transmembrane actin-associated nuclear (TAN) lines that are anchored by lamin A (Luxton et al., Science, 2010; Folker et al, PNAS, 2011). We found that nuclear movement was inhibited in both fibroblasts from patients with HGPS and NIH3T3 fibroblasts expressing progerin. Importantly, both the mobility and nuclear movement defects were rescued by treating cells with farnesyltransferase inhibitors (FTI-276 and FTI-277) and cells expressing a CAAX mutant progerin that could not be farnesylated, exhibited normal nuclear movement. Two factors contributed to defective nuclear movement in progerin-expressing cells. First, TAN lines, which normally are fixed in place on the nucleus, moved over the nuclear surface, indicating a failure to anchor TAN lines. Unlike wild type lamin A, GFP-progerin accumulated in the TAN lines and moved with them, probably due to the association of progerin with the membrane rather than the lamina. Second, the velocity of rearward actin cable movement was dramatically reduced from 0.28 $\mu\text{m}/\text{min}$ in controls to 0.08 $\mu\text{m}/\text{min}$ in progerin-expressing cells. ZMPSTE24 is the metallopeptidase that specifically cleaves prelamin A. Mutations in ZMPSTE24 lead to an inability to process prelamin A and cause progeria and restrictive dermopathy. We found that nuclear movement was also impaired in embryonic fibroblasts from Zmpste24-null mice and this defect was rescued by FTI-277 treatment. Zmpste24-null cells showed defects in cell spreading and failed to polarize and initiate cell migration. We conclude that both the abnormal processing of prelamin A and accumulation of progerin result in defective nuclear movement and cell migration in fibroblasts and suggest that these defects may contribute to the early aging symptoms of HGPS.

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Board Number: B479

LINC COMPLEXES AND THE B-TYPE LAMINS IN MAMMALIAN CONE PHOTORECEPTOR DIFFERENTIATION AND HOMEOSTASIS.

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Linkers of the Nucleoskeleton to the Cytoskeleton (LINC complexes) are macromolecular assemblies that span the whole nuclear envelope to physically connect chromatin and nuclear lamina to cytoskeletal components of the cytoplasm. We previously showed that LINC complexes are essential for the apical localization of cone photoreceptors nuclei. Here, using an improved transgenic mouse model, we show that cone nuclei mislocalization on the basal side of the outer nuclear layer affects cone pedicle morphology, synaptic transmission to secondary neurons as well as chromophore recycling to photosensitive cone outer segments. By contrast, the development and intrinsic phototransduction capabilities of the inner/outer segment interface remained unaffected. Because B-type lamins, which are major components of the nuclear lamina, directly interact with LINC complexes, we also examined their role in cone photoreceptor nuclei positioning. In one-month old retinas, the positioning of cone

nuclei was not affected upon genetic inactivation of LMNB1 and LMNB2 at postnatal day 6. As it turned out, our experiments directly demonstrated that lamin B1 and lamin B2 are very long-lived proteins whose clearance takes about 5 months. Despite the widely accepted assumption that B-type lamins are essential for cell viability, the morphology, function and viability of 9-month-old cone photoreceptors devoid of B-type lamins were unaffected. However, their nuclei were scattered on the apical side of the outer nuclear layer, a phenotype that was accompanied by the mislocalization of Sun1, an essential component of LINC complexes, from the nuclear envelope of cone nuclei.

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Board Number: B480

Initial characterization of DdMan1, the first LEM protein identified in *Dictyostelium discoideum*.

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Man1 is an integral protein of the inner nuclear membrane. It belongs to the LEM (Lap2, emerin, Man1) domain protein family based on a ~40 amino acids long LEM-domain motif at the amino-terminal region (Nicole Wagner, 2007, *Int Rev Cytol.*). We have identified a Man1 homologue protein in the eukaryotic organism *Dictyostelium discoideum*. Like higher eukaryotic LEM2 and MAN1, DdMan1 contains a N-terminal LEM motif, two predicted transmembrane segments and a C-terminal MAN1-Src1p C-terminal (MSC) domain (Feng Lin, 2005, *Hum Mol Genet*). Detergent extraction experiments suggest DdMan1 is an integral nuclear membrane protein. Similar to previously identified *Dictyostelium* nuclear envelope protein DdSun1 and the *Dictyostelium* lamin DdNE81, DdMan1 also localizes to the nuclear envelope throughout the whole cell cycle (Irene Schulz, 2009, *EJCB*; Anne Krüger, 2011, *MbOC*) (like yeasts and others lower eukaryotes, *Dictyostelium discoideum* undergoes a closed mitosis in which the nuclear envelope remains intact throughout the cell cycle). Surprisingly, fluorescence microscopy shows that DdMan1 only partially co-localizes with DdNE81, indicating the possible existence of micro-domains in the *Dictyostelium* nuclear envelope. When the first 646 amino acids, containing the LEM domain and the first transmembrane domain, of DdMan1 is overexpressed, several phenotypes were observed. 1) The nuclear envelope forms protrusions that extend into the cytosol. Fluorescence recovery after photobleaching (FRAP) experiments indicate that these are stable structures. 2) The nuclear envelope becomes grossly distorted in comparison to its normally oval or round appearance. 3) Cytoplasmic bridges that stain positive for both alpha-tubulin and DdMan1 appeared between adjacent cells. Taken together, the nuclear envelope phenotype indicates an important role of DdMan1 in nuclear envelope structure and organization.

P344**Board Number: B481****How forces generated in the cytoplasm are dissipated across the nucleoskeleton to move nuclei.**C.R. Bone¹, E.C. Tapley¹, P. Kuehnert¹, M. Gorjanacz^{2,3}, D.A. Starr¹;¹MCB, University of California Davis, Davis, CA, ²European Molecular Biology Laboratory (EMBL), Heidelberg, Germany, ³Therapeutic Research Group Oncology, Bayer HealthCare Pharmaceuticals, Berlin, Germany

Nuclear migration is a critical aspect of many developmental and cellular processes including fertilization, cell polarization, and differentiation. Disruption of proteins at the nuclear membrane responsible for nuclear migration leads to a class of diseases known as laminopathies, which includes Hutchinson-Gilford Progeria Syndrome and Emery-Dreifuss Muscular Dystrophy. For nuclear migration to occur, a bridge termed the LINC complex (for linker of the nucleoskeleton and the cytoskeleton), forms across the nuclear envelope. In *C. elegans*, the nuclear envelope bridge that functions in nuclear migration is made up of the KASH protein, UNC-83, which is recruited to the outer nuclear membrane by the SUN protein, UNC-84, in the inner nuclear membrane. This complex is thought to transduce forces generated by microtubule motors in the cytoplasm, across the nuclear envelope, and to the nucleoskeleton where the force is dissipated throughout the nucleus. We show that *C. elegans* lamin, LMN-1, is required for nuclear migration and interacts with the amino terminus of the SUN protein, UNC-84. This interaction is weakened by the UNC-84(P91S) mutation previously found to partially disrupt nuclear migration. Live imaging of nuclear migration in UNC-84(P91S) animals shows many nuclei migrate normally. However, other nuclei fail to initiate migration, while a third class of nuclei initiate migration before subsequently failing prior to completion. Our data also suggest a role for the NET5/Samp1/Ima1 homolog during nuclear migration in *C. elegans*. Interestingly, unlike in mammalian cells, *C. elegans* SAMP-1 does not require lamin for nuclear envelope localization. Additionally, RNAi experiments demonstrated SAMP-1 plays roles in organizing the mitotic spindle in the early embryo. Our working model is that the N-terminus of UNC-84 directly binds lamin to allow the transfer of force from the LINC complex to the nucleoskeleton where it is dissipated by a complex of proteins. Other inner nuclear membrane proteins, including SAMP-1, also function in these processes.

P345**Board Number: B482****The generation of topological order in the nuclear lamina - The four lamin systems.**K. Maass¹, T. Kolb², H. Herrmann¹;¹German Cancer Res Ctr, Heidelberg, Germany, ²University Erlangen-Nuernberg, Erlangen, Germany

Nuclear intermediate filament proteins (lamins) organize the interface between the nuclear envelope and chromatin. The importance of this structural network, the lamina, for cellular physiology becomes obvious by the reported 825 sequence variants in lamin genes linked to 41 associated diseases (www.interfil.org). In man, lamin B1 and lamin B2, are constitutively found within the nuclear lamina, whereas lamin A and its smaller splice form lamin C are differentially expressed during embryonic development. A-type lamins are in addition found in the nucleoplasm in the form of small soluble complexes. Notably, lamin A and lamin C do completely segregate, both in the soluble and the lamina-bound form despite the fact that their amino acid sequence is identical within the coiled coil domain that is mediating dimerization (1). In addition, we can show that lamin B1 and lamin B2 are kept segregated in cells. Hence the question arises how this order is established and maintained through the expansion of the nucleus during interphase and what the functional consequences of the coexistence of the four lamin systems are. We have studied the composition of the nuclear lamina by ectopic expression of fluorescently tagged A-type lamins in various stably transfected cell lines. We performed an integrated approach using fluorescence microscopy, differential cell extraction with the subsequent GFP-specific “pull-down” and separation of individual cell fractions by sucrose gradient centrifugation followed by Western blotting. We find that the GFP-lamin A chimeras distribute to different cell fractions than the endogenous A-type lamins. In contrast to endogenous lamin A, the majority of GFP-lamin A was extracted with buffers of both low and high detergent concentrations. Furthermore, when the GFP-lamin A chimera was precipitated with “GFP-binder” only minor amounts of the endogenous lamin A and lamin C were co-isolated indicating that they did not associate in the cell with one another, in particular did not form coiled coils. A separation by sucrose gradient centrifugation revealed that the complexes formed by GFP-lamin A differed in size from those recovered with the endogenous lamin A complexes. From this results we can conclude, that the nucleus provides a complex set of molecular mechanisms that mediates the functional organization of the four major lamin polypeptides as distinct structural entities in human cells.

(1) Kolb T, Maaß K et al. (2011) Lamin A and lamin C form homodimers and coexist in higher complex forms both in the nucleoplasmic fraction and in the lamina of cultured human cells. *Nucleus* 2, 425-433.

P346**Board Number: B483****Structural Analysis of the Nuclear Lamina by Nanoscale Resolution Microscopy.**T. Shimi¹, Y.G. Turgay², M. Kittisopikul³, A.E. Goldman¹, S.A. Adam¹, Y. Guo⁴, K. Jaqaman³, Y. Zheng^{4,5}, O. Medalia², R.D. Goldman¹;¹Department of Cell and Molecular Biology, Northwestern University, Feinberg School of Medicine, Chicago, IL, ²Department of Biochemistry, University of Zürich, Zurich, Switzerland, ³Department of Biophysics, University of Texas Southwestern Medical Center, Dallas, TX, ⁴Department of Embryology, Carnegie Institution for Science, Baltimore, MD, ⁵Department of Biology, Johns Hopkins University, Baltimore, MD

In mammalian cells, nuclear lamins are the major components of the nuclear lamina which underlies the nuclear envelope (NE). They are subdivided into the A-type lamins (LA and LC) and the B-type lamins (LB1 and LB2). However, the structural organization of each of these lamin isoforms within the lamina remains unclear. The elucidation of the 3D structural organization of each type of lamin is essential for understanding their structure and functional interactions with peripheral heterochromatic regions of interphase chromosomes. In order to study this, 3D Structured Illumination Microscopy (3D-SIM) combined with computational image analysis was performed on WI-38 human diploid fibroblasts and mouse embryonic fibroblasts (MEFs). Interestingly, LA, LB1, LB2 and LC form separate fibrillar structures which appear to overlap at their ends to form complex meshworks. Using LB1 null (*Imnb1*^{-/-}) MEFs, we also show that the lamina meshwork formed by LA/C and LB2 fibrils becomes abnormally enlarged compared to WT MEFs. The enlarged complexes of these fibrils align with peripheral elements of heterochromatin as determined by staining with anti-H3K27me3. These results suggest that LB1 regulates both the normal structure of the lamina and the normal structural links to peripheral heterochromatin. To go one step further in determining nanoscale structures of the lamina, we have used vimentin null MEFs (*Vim*^{-/-} MEFs), the nucleus of which lies in close proximity to the plasma membrane due to the loss of the juxtannuclear intermediate filament cage. This allows us to perform Stochastic optical reconstruction microscopy (STORM) with total internal reflection fluorescence microscopy (TIRF) to achieve even higher resolution images of the lamina in situ. These nuclei can also be imaged in situ by advanced cryo-electron tomography which reveals that the major filamentous structures in the lamina are not 10nm in diameter but rather are about 5 nm in diameter. We propose that these lamin nanofilaments probably associate laterally to form the lamin fibers resolved by 3D-SIM and STORM. These results are being confirmed by the observation that lamin null MEFs in which vimentin expression is silenced and *Vim*^{-/-} MEFs stably overexpressing lamins exhibit significant changes in number of lamin nanofilaments. Our results demonstrate that the lamin fibers assemble into a complex hierarchical network which changes its structure and function regionally within the nuclear lamina. This work is supported by National Institutes of Health grants (GM106023) and (CA03176).

P347**Board Number: B484****The Role of A Type Lamins in Genome Organization.**X. Wong¹, J.C. Harr¹, K.L. Reddy²;¹Biological Chemistry, Johns Hopkins University School of Medicine, Baltimore, MD, ²Biological Chemistry and Center for Epigenetics, Johns Hopkins University School of Medicine, Baltimore, MD

A type lamins, in particular lamins A and C, are spliceoforms of the LMNA gene. For the majority of the coding sequence, Lamin A and C are identical and as such, these two spliceoforms have been considered similar and mostly studied together. We have designed tools to specifically study the roles of these two spliceoforms independently and have uncovered differential roles of lamin A and C, at least in genome organization. We suggest that understanding the specific roles these two spliceoforms play in establishing proper genome organization will provide insights to organizational changes that occur during development and diseases such as Hutchinson-Gilford Progeria Syndrome. Additionally, we propose that other cellular functions might also be carried out differently by these two spliceoforms. As such, understanding the specific roles lamins A and C play in establishing proper cellular functions will potentially provide insights for treating the myriad of diseases of the nuclear envelope or the Laminopathies.

P348**Board Number: B485****Identifying a novel role for nuclear lamins in controlling nuclear shape changes in response to feeding.**E.M. Zeituni¹, X. Zheng¹, B. Caldwell¹, Y. Zheng^{1,2}, S.A. Farber^{1,2};¹Department of Embryology, Carnegie Institution for Science, Baltimore, MD, ²Department of Biology, Johns Hopkins University, Baltimore, MD

The nuclear lamina is a complex structure whose composition and interactions appear to dynamically change through development and across tissues. Due to its complexity and variability, fundamentally understanding the biology of the nuclear lamina in vivo remains largely out of reach. Using zebrafish larvae, we are able to observe remarkable morphological changes in intestinal epithelial cell nuclei during feeding. These enterocytes respond rapidly and robustly to the presence of nutrients, especially lipids. Morphological responses include increases in size, expansion of endoplasmic reticulum, fusion of mitochondria, lysosomal reconstruction, and the formation of lipid droplets. Interestingly, we observed a novel phenomenon: the rapid and reversible blebbing of the nuclear periphery upon feeding. This dramatic structural change of the nucleus is coupled with robust and widespread transcriptional changes. Since the nuclear lamina helps regulate both the shape of the nucleus and the transcription of genes, we examined the role of nuclear lamins in these physiologically responding enterocytes. Using zebrafish that express a human mutant allele of lamin A, called progerin, we studied the impact of a disrupted nuclear lamina on the morphology and transcriptional responses of enterocytes. Based on our

findings we have constructed a model where the nuclear lamina plays a novel role in cellular responses to nutrients.

P349

Board Number: B486

Defective nuclear movement in lamin A deficient cells is rescued by reducing activity of Erk1/2 and Rho signaling.

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In NIH3T3 fibroblasts, the serum factor lysophosphatidic acid (LPA) stimulates actin retrograde flow to power the rearward movement of the nucleus to polarize cells for migration. Retrogradely moving actin cables are attached to the nucleus through LINC complexes of nesprin-2G and SUN2 that are thought to be anchored by lamin A (Luxton, G. et al., *Science*, 2010; Folker E., et al., *PNAS*, 2011). Expression of variants of lamin A that cause muscle dystrophies disrupts nuclear movement in fibroblasts and also causes upregulated Erk 1/2 activity (Muchir, A., et al., *JCI*, 2007). As reducing Erk 1/2 activity with MEK 1/2 inhibitors ameliorates dystrophic symptoms in a mouse model (Muchir, A. et al., *HMG*, 2009), we tested the relationship between Erk 1/2 activity and nuclear movement in NIH3T3 fibroblasts. Treatment of lamin A knockdown fibroblasts or fibroblasts expressing the lamin A disease variant, lamin A H222P, with MEK1/2 inhibitors (AZD 6244 and PD98059) rescued the defect in nuclear movement. Consistent with these results, hyperactivation of Erk 1/2 activity in NIH3T3 fibroblasts by expressing a constitutively active MEK1 S218/222D inhibited nuclear movement. The rescued nuclear movement by MEK 1/2 inhibitors in lamin A deficient fibroblasts had characteristics, including actin-dependence, similar to wild type cells. LPA also stimulates Erk1/2 activity with peak activation occurring by 5 min. Treatment of wild type fibroblasts with AZD6244 to block this Erk1/2 activation revealed that nuclear movement was initiated and completed earlier without affecting the rate of actin retrograde flow. Erk 1/2 activates Rho through GEF1-H1 (Fujushiro, S., et al., *BBRC*, 2008), so we tested whether Erk1/2 may mediate its effects on nuclear movement through Rho. Mild inhibition of Rho by C3 toxin rescued the nuclear movement defect in lamin A knockdown fibroblasts, whereas hyperactivation of Rho by CN03 toxin inhibited nuclear movement in wild-type fibroblasts. Furthermore, mild inhibition of Rho kinase by Y27632 also rescued the nuclear movement defect. Our results reveal that Erk1/2 and Rho are negative regulators of nuclear movement and suggest that lamin A may contribute to nuclear movement by maintaining Erk1/2 and Rho at low levels permissible for nuclear movement.

P350**Board Number: B487****Lamins and emerin determine mobility of SUN proteins at the nuclear envelope.**P. Isermann¹, B. Nguyen², J. Lammerding³;¹Weill Institute, Cornell University, Ithaca, NY, ²Cornell University, Ithaca, NY, ³Department of Biomedical Engineering, Cornell University, Ithaca, NY

Mutations in the nuclear envelope proteins lamin A/C, emerin, nesprin, and Sun1/2 can cause muscular dystrophy and dilated cardiomyopathy, but the molecular disease mechanism remains incompletely understood. SUN proteins at the inner nuclear membrane and nesprins at the outer nuclear membrane form the linker of the nucleoskeleton and cytoskeleton (LINC) complex, which is essential for nuclear positioning and other cell functions. Since LINC complex proteins directly interact with lamins and nesprins, mutations in any of these proteins may disrupt nucleo-cytoskeletal coupling and contribute to the disease mechanism.

To test the hypothesis that mutations or loss of lamins A/C or emerin can impair anchoring of the LINC complex at the nuclear envelope, we used fluorescence recovery after photobleaching (FRAP) of fluorescently tagged Sun1, Sun2, and Samp1 to investigate their mobility. In case of Sun1 and -2, we used full-length constructs as well as truncated versions lacking the nesprin-binding site to prevent confounding effects from interactions with the cytoskeleton via nesprin. Experiments were carried out in mouse embryonic fibroblasts lacking specific lamin isoforms, the lamin A processing enzyme Zmpste24, and in cells expressing specific emerin mutants.

Cells lacking both lamins A and C had higher mobility of Sun1 and -2 compared to wild-type controls. Cells expressing lamin A but lacking lamin C had normal mobility of Sun1, but not Sun2, whereas mobility of both Sun1 and -2 was increased in cells expressing only lamin C and no lamin A. In emerin- and lamin B1-deficient cells, Sun1 mobility was comparable to wild-type cells, but Sun2 was more mobile, similar to the results in lamin A/C-deficient cells. Expression of emerin mutants that cause impaired interaction of emerin with actin did not affect Sun2 mobility. The mobility of Samp1, which is a recently discovered binding partner of Sun2 and emerin, was increased in lamin A/C-, lamin B1-, and emerin-deficient cells. Interestingly, lamin B2 deficiency increased the mobility for Sun1/2 but not Samp1. In contrast, the emerin mutations resulted in higher mobility of Samp1, suggesting that the interaction of emerin with Samp1 may be mediated by actin. Deletion of Zmpste24, resulting in permanently farnesylated lamin A, reduced the mobility of Sun1.

Our results suggest that Sun1 and Sun2 interact with distinct nuclear lamina proteins, indicating different functions of lamin proteins. Sun1 mobility was primarily determined by interaction with lamin A, whereas Sun2 mobility was affected by any deletion of a component of the lamina, but was independent of lamin A farnesylation. Mutations in lamins and emerin may affect LINC complex function by altering SUN protein mobility.

P351**Board Number: B488****Regulation of the transcriptional coactivator MKL1 by the nuclear lamina.**M. Sledd¹, C. May¹, T. Carroll²;¹Cell Biology, Yale University, New Haven, CT, ²Dept. of Cell Biology, Yale University, New Haven, CT

The physical characteristics of animal cells, including shape and motile properties, are largely determined by the actin cytoskeleton. Diverse chemical or mechanical stimuli activate Rho-family GTPases which allow cells to rapidly alter their physical characteristics in response to changes in their microenvironment by promoting actin filament assembly. The transcriptional coactivator Megakaryoblastic Leukemia 1 (MKL1) senses monomeric actin (G-actin) levels. MKL1 is localized to the cytoplasm when bound to G-actin but translocates to the nucleus in response to actin polymerization. Once in the nucleus, MKL1 binds to Serum Response Factor (SRF) and activates target gene expression. Thus, MKL1 converts a rapid change in actin assembly into a lasting pattern of gene expression. Recently, components of the nuclear lamina, including Lamin A/C and Emerin, were shown to facilitate MKL1 localization (Ho et al, 2013). We have found that Man1, an inner nuclear membrane protein, also regulates MKL1. Man1 did not affect MKL1 nuclear translocation, however, transcription of MKL1-SRF target genes decreased with Man1 depletion. These results suggest that Emerin facilitates MKL1 nuclear localization whereas Man1 independently promotes MKL1 activity in the nucleus. We are examining the specific mechanisms by which these proteins affect MKL1 localization and activity.

Ho, C. Y. Jaalouk, D. E., Vartiainen, M. K. & Lammerding, J. Lamin A/C and emerin regulate MKL1-SRF activity by modulating actin dynamics. *Nature* 497, 507-511 (2013).

P352**Board Number: B489****Regulation of Lamin A assembly and abundance by SRF/Mkl1.**T. Carroll¹, K. Thakar¹;¹Dept. of Cell Biology, Yale University, New Haven, CT

The nuclear lamina is a dense protein meshwork that lines the nucleoplasmic face of the nuclear envelope in metazoan cells. Composed of a polymeric network of intermediate filament-like proteins called lamins, the nuclear lamina directly interacts with the genome and regulates virtually all aspects of DNA metabolism. The mechanical properties of the cellular microenvironment regulate the abundance and assembly state of Lamin A, which in turn, influences cell identity and function. The mechanosensitive signaling pathways that control Lamin A abundance and assembly state, however, have not been fully delineated. Here, we show that activation of the mechanosensitive Serum Response Factor (SRF)/Megakaryoblastic Leukemia 1 (Mkl1) transcription factor/coactivator complex leads to an increase in the abundance and decrease in the solubility of Lamin A. SRF/Mkl1 controls the expression of

genes that promote actin stress fiber assembly and cell contractility. Interestingly, Lamin A was recently shown to be required for SRF/Mkl1-dependent gene expression. We hypothesize that the requirement for Lamin A in the regulation of Mkl1/SRF activity reflects a cellular mechanism to protect genome integrity; stress fiber assembly is expected to increase the magnitude of an actin-dependent force that is applied directly to the nucleus, which would be particularly harmful in cells with defects in nuclear lamina structure. To test this hypothesis, we have developed stable cell-lines that express a constitutively active Mkl1 transgene, which should bypass the requirement for Lamin A in the activation of the SRF/Mkl1 complex. We are currently exploring the consequences for genome integrity of activating SRF/Mkl1 in the absence of a fully functional nuclear lamina.

P353

Board Number: B490

As the beating heart stiffens in development, so does the nuclear lamina.

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The heart is the first organ to develop in vertebrate embryos and is a mechanically stressed pump that is susceptible to defects in structural proteins of the extracellular matrix, cytoskeleton, and even the nucleus. We first show that the nucleus beats in the intact embryonic heart and that nuclear beating in vitro is regulated by matrix stiffness. However, it has been unclear when the nuclear structure protein Lamin-A turns on, which seems important because *Lmna* knockout mice are known to exhibit 'developmental defects of the heart' and die shortly after birth. We show that nuclear lamins parallel the development of mechanical stiffness and stress in normal embryos. While early embryos are well-known to be uniformly soft, our measurements using a chick embryo model show that the heart begins to stiffen almost immediately following initial differentiation, with tissue stiffness E of 1-2 kPa at embryonic day 4 (E4) increasing significantly by E6. This is accompanied by an increase in Lamin-A levels and comparatively constant Lamin-B expression with Lamin-A:B stoichiometry already known to function as a mechanosensor in adult tissue [Swift *Science* 2013]. Proteomic analyses of lamin perturbations at different stages of development likewise reveal nuclear lamins as potential embryonic stress sensors that mechanically link to cytoskeleton and the matrix, potentially feeding back to cytoskeletal maturation and differentiation.

P354**Board Number: B491****Microtubules contribute to the abnormal nuclear morphology in Hutchinson Gilford Progeria Syndrome.**Z. Tariq¹, H. Zhang¹, W. Losert², K. Cao¹;¹Dept of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD, ²Dept of Physics, University of Maryland, College Park, MD

HGPS Syndrome is a genetic disorder characterized by rapid, premature aging and has been an area of interest due to its potential as a model system for normal aging. One of the hallmarks of HGPS is an abnormal nuclear shape which appears to hold clinical significance in itself, especially since a similar phenotype arises in cancer. Previous studies of this abnormality in HGPS have pointed to the accumulation of a mutant protein, progerin, as the major culprit. However, the potential interplay between the nucleus and the cytoskeleton has not been fully explored. Here we perturbed the microtubule network in both HGPS and control fibroblast cells through either microtubule drug treatments (nocodazole and taxol) or through an siRNA treatment against SUN1, an adaptor protein connecting the microtubule network and nuclear scaffolding. The nuclei of these cells then underwent two types of imaging: fluorescent microscopy and live cell spinning disc confocal microscopy. The resulting movies/images were then analyzed by an in-house MATLAB program, thereby allowing for a sensitive quantification of the nucleus. Surprisingly, we find that the microtubule network plays a positive role in normal cells and a negative role in HGPS cells in regulating nuclear shape.

Nucleocytoplasmic Transport 1**P355****Board Number: B492****The integrity of a transcriptional pulse from identical gene alleles is governed by regulated amounts of nuclear signaling factors.**A. Kalo¹, I. Kanter¹, A. Sharga¹, H. Tzemach¹, R. Singer², T. Lionnet³, Y. Shav-Tal¹;¹Faculty of Life Sciences, Bar-Ilan University, Ramat Gan, Israel, ²Albert Einstein College of Medicine, Bronx, NY, ³Janelia Farm, Ashburn, VA

Imaging of transcription in living cells using fluorescence microscopy has become an important approach for understanding nuclear gene expression dynamics. Functional interactions that combine signaling pathways and transcription factors are a mechanism used by cells to govern gene-specific responses to various stimuli. However, it is unknown if identical alleles in the nucleus respond similarly or differently to such signaling pathways, and whether the transcriptional pulse is coordinated between the alleles. Such questions can be approached using live-cell systems in which transcription from single alleles can be followed and quantified in real-time and at high resolution. The transcriptional response of the β -

actin gene to extra-cellular stimuli is a paradigm system for transcription factor complex nuclear assembly and regulation. Serum induction leads to a precisely timed pulse of β -actin transcription occurring within minutes of serum addition. We examined how the serum-induction signaling pathway governs the efficacy of the induced transcriptional pulse from several endogenous alleles of β -actin, using the MS2-tagging method for following mRNA transcription in single living cells. Our study focused on the nuclear serum response factor (SRF), the MAL transcription factor that translocates to the nucleus upon serum addition, and the actin protein. For instance, we found that lowering SRF levels led to loss of the transcriptional pulse, including a disordered response time, and reduction of activation coordination between the alleles. In contrast, reducing actin protein levels revealed a positive feedback response from the cytoplasm to the nucleus, resulting in stronger allele activation, a prolonged transcriptional response, and increased coordination between the alleles. This study reveals additional important aspects of nuclear dynamics, namely the very rapid time-frames of signal propagation from the cell membrane to the nucleus and RNA Pol II assembly on the promoter, RNA Pol II elongation rates, and nucleo-cytoplasmic transport kinetics of the mRNA wave generated by the pulse. This study on single alleles in living cells demonstrates that transcriptional pulse fidelity requires regulated amounts of nuclear signaling proteins, and that perturbations in factor levels in the nucleus eliminate the sharp pulse, resulting in either toning down or exaggeration of the transcriptional response.

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Board Number: B493

Eukaryotic translation elongation factor 1 alpha 1 is Involved in the Nucleocytoplasmic Transport of Proteins Carrying an Expanded Polyalanine Tract.

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Polyalanine (poly-Ala) diseases are caused by expansion of translated GCX triplet nucleotide sequences encoding for poly-Ala tract in disease proteins. To date, nine human disorders are found to associate with poly-Ala tract expansion, including synpolydactyly type II, cleidocranial dysplasia, holoprosencephaly type 5, hand-foot-genital syndrome, blepharophimosis epicanthus inversus syndrome, mental retardation with growth hormone deficiency, Partington syndrome, congenital central hypoventilation syndrome and oculopharyngeal muscular dystrophy. Of note, eight out of nine poly-Ala disease genes encode transcription factors with important roles during development and differentiation. Previous studies demonstrate that wildtype unexpanded poly-Ala proteins localize to the cell nucleus while their respective expanded poly-Ala disease proteins primarily localize to the cytoplasm. The mislocalization of expanded polyA disease proteins causes cellular transcriptional dysregulation, which subsequently leads to cell dysfunction. Our laboratory found that expanded poly-Ala domain possesses nuclear export signal (NES) activity. By means of glutathione-S-transferase (GST) pull-down assay followed by mass spectrometry, we identified the eukaryotic translation elongation

factor 1 alpha 1 (eEF1A1) as an interacting protein of expanded poly-Ala-containing protein. Knockdown of *eEF1A1* expression suppressed expanded poly-Ala protein nuclear export. In addition, knockdown of *eEF1A1* restored the cellular function of poly-Ala-containing protein. Elucidating the mechanism behind expanded poly-Ala protein nuclear export will provide further insights into the pathogenesis of poly-Ala diseases.

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P357

Board Number: B494

Signals and blockers for karyopherin-mediated nuclear transport.

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Each of the ~20 different Karyopherin- β proteins (Kaps; Importins and Exportins) that mediate the majority of nuclear-cytoplasmic protein transport, recognizes distinct nuclear localization or export signals (NLSs or NESs) in their cargos. The classical-NLS, the PY-NLS, the lysine-rich K-V/I-x-K-x1,2-K/H/R NLS and the RS-domain NLS are recognized by the Imp α -Imp β heterodimer, Kap β 2 (or Trn-1), Kap121 (and its human homolog Imp-5) and TrnSR (or TNPO3), respectively. For nuclear export, only the classical NES recognized by CRM1 (or Xpo1) is currently known. NLSs and NESs for many Importins and Exportins remain uncharacterized. Core histones were previously shown to be imported by several Importins, with Importin-4 as the primary importer, but it was unclear if different Importins recognize different NLSs or a single common NLS within the histone tails. Analysis of core histones showed different distribution of binding energies within their N-terminal tails with different Importins. Two Kap pathways have recently been shown to be important for human diseases: nuclear import of RNA binding protein FUS by Kap β 2, and nuclear export by CRM1. The PY-NLS of FUS is heavily mutated, causing Kap β 2-mediated nuclear import defects in familial amyotrophic lateral sclerosis (ALS) patients. We had previously explained how the PY-NLS of FUS interacts with Kap β 2 and now expands knowledge of interactions to full length FUS and its other ligands. Finally, many tumor suppressor/growth regulatory proteins, which act in the nucleus to prevent genesis of neoplastic clones, are cargos of the Exportin CRM1. Activated oncogenic signaling pathways can alter these proteins causing inappropriate access to CRM1, mislocalization to the cytoplasm and loss of tumor suppressor activities. Small molecule CRM1 inhibitor KPT-330/Selinexor (currently in Phase 1 and 2 clinical trials for various cancers) retains dysregulated tumor suppressor proteins in the nucleus, reinitiating their antitumor, growth suppressive and pro-apoptotic activities and causes apoptosis of cancer cells while sparing normal cells. Tolerance of KPT drugs is greatly improved over the older lactone polyketide CRM1 inhibitor Leptomycin B (LMB), underscoring the importance of understanding differences in their mechanisms of action. We have shown that mechanisms of action for KPTs versus LMB are different despite both drugs binding covalently to the same CRM1 site.

P358**Board Number: B495****Recognition of the PY-Less PY-NLS by Kap β 2.**M. Soniat¹;¹Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX

Import-Karyopherins or Importins bind nuclear localization signals (NLSs) to mediate the transport of proteins into the cell nucleus. Karyopherin β 2 (Kap β 2), also known as Transportin-1, is an Importin that imports various RNA binding proteins into the nucleus. Kap β 2 recognizes a targeting signal named the PY-NLS that lies within its cargos, to target them through the nuclear pore complex. PY-NLSs can be described by a set of rules, which include 1) structural disorder, 2) overall positive charge, and 3) weakly conserved sequence motifs composed of a loose N-terminal hydrophobic or basic motif and a C-terminal RX₂₋₅PY motif. Several Kap β 2 cargos such as core histones H3 and H4 do not seem to contain any recognizable PY-NLSs, and their interactions with the Importin have not been characterized. Previous biochemical studies suggest that the N-terminal tails of H3 and H4 might bind Kap β 2 in a site distinct from the PY-NLS binding site. We are using structural and biochemical analysis of Kap β 2 bound to histone tails to better understand interactions with these atypical Kap β 2 cargos. We show that the histone tails bind with high affinities to the PY-NLS binding site of Kap β 2. Our results reveal a new class of PY-NLS variants that do not contain the P-Y or homologous P- ϕ (where ϕ is a hydrophobic amino acid) motif.

P359**Board Number: B500****Exploring the Complete Spectrum of CRM1 Cargoes.**K. Kirli¹, S. Karaca², H. Urlaub², D. Görlich¹;¹Cellular Logistics, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany, ²Bioanalytical Mass Spectrometry, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany

CRM1 is an essential, highly conserved RanGTPase-driven exportin. It mediates biosynthetic nuclear export, counteracts leakage of cytoplasmic proteins into the nucleus, and mediates many instances of regulated nuclear export. CRM1 recognizes its cargoes through nuclear export signals (NESs), which are 10-15 residues long and contain 4-5 hydrophobic residues (Φ residues) that dock into complementary binding pockets of the exportin. So far, more than 250 CRM1 cargoes have been identified from various species; however, this probably represents just a small fraction of the actual cargo pool. To explore the complete spectrum of CRM1 dependent cargoes, we developed an optimized CRM1 affinity chromatography method. Indeed this strategy revealed hundreds of new CRM1 cargo candidates each from *S. cerevisiae*, *Xenopus laevis* and human cells. These candidates might be the direct CRM1 binders or the associated partners of CRM1 cargoes. Analysis of primary protein structure, in principle, could reveal direct CRM1 cargoes by identifying a functional NES. However, it is still a major challenge to

predict NESs with high sensitivity and accuracy. We now present a new bioinformatics NES prediction method that is based on (i) the recent crystal structures of different NES peptides in complex with CRM1. (ii) It considers new experimental data correlating systematic mutations in NES sequences with export activity and CRM1-binding strength, which suggest that a greater variety of residues are allowed at Φ positions and that the inter Φ spacers impose more constraints than previously thought. (iii) It also considers that functional NESs must be solvent exposed; it thus includes a filtering of initial hits with domain and disorder prediction tools. We validated this prediction tool with already known NESs as controls and also verified cargoes with so far unidentified NESs. With that, we were able to predict NESs of human eIF2 β and *S. pombe* Rna1p, and these predictions perfectly matched subsequent experimental validations.

P360

Board Number: B501

Nuclear size control depends on spatial information within the cell in *Xenopus laevis* egg extracts.

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Nuclear size changes dynamically during development and has long been observed to correlate with cell size. However, the underlying molecular mechanisms controlling nuclear scaling have remained largely unknown. Here, we combine an *in vitro* cell-free system of *Xenopus laevis* egg extract with microfluidic devices to systematically analyze the effect of spatial constraints. The speed of nuclear expansion depended on the available space surrounding the nucleus up to a threshold volume in the nanoliter range, herein referred to as the nuclear domain. Under spatial constraints smaller than the size of nuclear domain, the accumulation of membranes around the nucleus was limiting nuclear expansion. Detailed biochemical studies revealed that mechanisms sensing the space are not mainly regulated by simple diffusion of nuclear components, but rather by microtubules accumulating membranes via the motor protein dynein. This mechanism helps understanding how spatial information surrounding the nucleus, like positioning of the nucleus inside the cell, can control the nuclear expansion *in vivo*.

P361**Board Number: B502****Nuclear Import Competence during Differentiation of Multinucleated Skeletal Muscle Cells.**A.A. Cutler¹, J.B. Jackson², G.K. Pavlath³;¹BCDB, Emory University, Atlanta, GA, ²Surgery, Boston University, Boston, MA, ³Pharmacology, Emory University, Atlanta, GA

Skeletal muscle is critical for survival and quality of life. Proper gene expression is essential for myogenesis and maintenance of skeletal muscle. During myogenesis, myoblasts differentiate into myocytes, which fuse to form mature myotubes containing thousands of nuclei. Proteins that regulate gene expression are synthesized in the cytoplasm and cross the nuclear pore to access their targets in the nucleus. To cross the nuclear pore, large proteins must be bound to a nuclear transport receptor, which recognizes a nuclear localization signal (NLS) within the cargo protein. While the mechanism of protein import is well characterized, how nuclear import is regulated in differentiation as muscle cells transition from being mononucleated to multinucleated and how alterations in nuclear import impact gene expression remain unknown. We examined nuclear import in cultured primary murine myotubes using an in vitro nuclear import assay and found that a subset of nuclei were incompetent to import a protein reporter containing the most common NLS, the classical NLS (cNLS). When cNLS import-competence was investigated across myogenesis, we found that, while almost all myoblast nuclei are cNLS import-competent, as myoblasts differentiate into myocytes, only a low percentage of myonuclei are cNLS import-competent. However, as myocytes develop into mature myotubes, cNLS import is restored. Pursuing the mechanism for these differences in import competence we found an overall increase in nuclear glycosylation during myogenesis and variation in the abundance and glycosylation of several nuclear pore proteins. These results suggest that modification of the nuclear pore could be a mechanism for selectively modulating nuclear import in differentiation.

P362**Board Number: B503****Active nuclear transport is disrupted before nuclear envelope breakdown during chemotherapy induced apoptosis in HeLa cells by 5-fluorouracil but not camptothecin and its derivatives.**R. Anderson¹, C. Campbell¹, S. Broskin¹, B. Colosimo¹, K. Resendes¹;¹Biology, Westminster College, New Wilmington, PA

Chemotherapeutic agents are used to activate apoptosis to eliminate cancer cells. The stepwise mechanism they activate involves caspase-regulated breakdown of the nuclear envelope to initiate DNA fragmentation. However, there is conflicting evidence on whether nuclear transport is specifically disrupted before the destruction of the nucleus. In this study, we explored the effects of four

chemotherapeutic drugs, 5-fluorouracil (5-FU), camptothecin, and its derivatives topotecan and irinotecan, on nuclear transport during apoptosis in HeLa cells. These drugs were selected because they activate different mechanisms of apoptosis (5-FU is extrinsic and the others intrinsic) and because 5-FU is often used in combination with the camptothecin derivatives during chemotherapy. The membrane impermeable DNA dye, propidium iodide (PI), was used to determine the timing of apoptotic nuclear envelope breakdown (NEB), which was confirmed by immunofluorescence of nuclear pore proteins. Based on the PI assays functioning nuclear transport was monitored prior to NEB via immunofluorescence of Ran, the major directional regulator of nuclear protein transport, which is normally at high concentrations in the nucleus. We found that 5-FU disrupted nuclear transport before NEB, whereas camptothecin and its derivatives only displayed cytoplasmic mislocalization of the Ran concurrent with NEB. This variation in the targeted disruption of nuclear transport between 5-FU and the camptothecin drugs may stem from a variation in intrinsic versus extrinsic apoptosis. Interestingly, combination of 5-FU with camptothecin did not induce an early disruption of nuclear transport whereas 5-FU with topotecan did in fact disrupt Ran localization before NEB. Future directions will include analysis of the 5-FU and irinotecan combination and investigations into the mechanism behind the variation between targeted disruption of nuclear transport versus maintaining transport until NEB, including profiling the activation of caspases in each scenario. Overall, our results suggest that active nuclear transport may be required in certain mechanisms of apoptosis whereas its disruption may be needed in others. Because regulation of nuclear transport is altered in many cancer types this intriguing new variation in the mechanism of apoptosis could possibly be exploited when determining chemotherapeutic agents in relation to the status of nuclear transport in cancer cells.

P363

Board Number: B504

Super-resolution microscopy study of the pre-ribosomal subunit nuclear export mechanism.

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The existence of the nuclear envelope (NE) in eukaryotic cells provides a barrier separating nascent pre-ribosomal subunits assembled in the nucleus from matured subunits functioning to translate proteins in cytoplasm. Nuclear pore complexes (NPCs) act as the sole gatekeeper for large and small pre-ribosomal subunits (pre-60s and pre-40s) exiting the nucleus. Hindered by the diffraction limit and insufficient temporal resolution of conventional light microscopy, the precise transport mechanism including the transport kinetics and the spatial transport routes for both large and small pre-ribosomal subunits through NPCs remains largely unknown. Here we combined an innovative super-resolution single-molecule microscopic approach, termed as single-point edge-excitation sub-diffraction (SPEED) microscopy, with Förster resonance energy transfer (FRET) to obtain real-time single-molecule trajectories as well as the three-dimensional transport pathways for both pre-60s and pre-40s in live HeLa cells. Our results reveal that both pre-ribosomal subunits conduct a fast-slow-fast diffusion pattern

through the three sub-regions of the NPC (nuclear side, central channel, and cytoplasmic side) during their export time of ~14 ms for pre-60S and ~8 ms for pre-40S. Remarkably, approximately two thirds of all NPC-interacting pre-60s and pre-40s subunits successfully exit the nucleus and enter the cytoplasm resulting in much higher transport efficiencies than protein cargos and mRNAs.

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Board Number: B505

Oxidative Stress Disrupts the Ran System Through Reversible Modification of Cysteine Residues in RCC1.

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The small GTPase Ran acts as a key regulator of nucleocytoplasmic transport within the nucleus by promoting import complex disassembly and export complex assembly. Both reactions result in nuclear export of Ran, which is re-imported into the nucleus via a dedicated receptor NTF2. Ran, therefore, undergoes rapid and continuous shuttling between the nucleus and cytoplasm. Ran displays a steady state distribution that is ~3-fold higher in the nucleus, which we refer to as the Ran protein gradient. Here, we describe biochemical approaches used to uncover a mechanism for how oxidative stress disrupts the Ran gradient. Treating cells with the thiol oxidant diamide results in disruption of the Ran protein gradient (p

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Board Number: B506

Intranuclear trafficking and export of single ribosomal subunits in mammalian cells.

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In eukaryotes, the assembly of ribosomes requires more than 200 RNA and protein cofactors. The exact role of many of these factors is not fully understood. Also, the dynamics of the ribosomal subunit synthesis and their complex intracellular processing, e.g. the intranuclear transport and export process of single subunits is largely unknown. The major difficulty to study these questions is the visualization of ribosomal subunits without interfering with their native behavior. Recently, we established a protein-based mRNA-labeling approach using an endogenous mRNA binding protein in the salivary gland cells of *Chironomus tentans* [1]. Now, we used a similar approach to fluorescently label ribosomal subunits in mammalian cells. Here we use Dim2/Pno1, a protein known to bind cotranscriptionally to the pre-40S-rRNA and to remain bound to it during maturation and export [2]. We created stable cell lines

expressing either Dim2-eGFP or -Snap fusion proteins under control of a tetracycline-repressor. For labeling the nuclear envelope we microinjected fluorescent NTF2, a transport factor being enriched at nuclear pores. Very sparse fluorescence labelling of the Snap-Tag was performed using nanomolar concentrations of SiR-Snap [3], what allowed extended intracellular visualization and tracking of single Dim2 and Dim2-labelled ribosomal subunits. The trajectories of single native ribosomal subunits could conveniently be followed using fast and sensitive fluorescence microscopy with HILO illumination. Since Dim2 accompanies the rRNA from the transcription site to the cytoplasm the route of transport, including the NPC passage, could directly be monitored in living cells with high space and time resolution. We found that the particles remained bound in the nucleolus for extended time periods before they diffused rapidly through the nucleoplasm and were exported across the nuclear envelope. Binding durations, diffusion constants and translocation times could quantitatively be determined.

[1] Siebrasse et al. (2012), Proc Natl Acad Sci USA 109: 9426-31

[2] Campbell & Karbstein (2011), PLoS One 6, e16194

[3] Lukinavičius et al. (2013), Nat Chem 5, 132-139

P366

Board Number: B507

Disordered Proteins Break the Size-Exclusion Rule of Nucleocytoplasmic Transport.

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Many intrinsically disordered proteins (IDPs) carry out important biologic functions in the nucleus; the selective mechanism of nucleocytoplasmic transport for IDPs is critical but not well understood. In this study we employed single-point edge excitation sub-diffraction microscopy to determine the transport kinetics and three-dimensional spatial transport routes for multiple natively unfolded endogenous proteins through the nuclear pore complex (NPC). Strikingly, the size criterion working for the nucleocytoplasmic transport of folded protein is not followed by IDPs; instead, the ratios between charged and hydrophobic amino acids in IDPs are the key criterion in the selectivity of NPC for IDPs.

P367**Board Number: B508****Molecular Determinants for mRNA remodeling at the Nuclear Pore Complex.**R.L. Adams¹, S.R. Wente¹;¹Vanderbilt University Medical Center, Nashville, TN

Eukaryotic mRNA is exported from the nucleus as a ribonucleoprotein particle (mRNP) coated with associated RNA binding proteins. Some of these factors assemble co-transcriptionally and dictate mRNA localization, function, and stability after export. Others are required specifically for export through nuclear pore complexes (NPCs) and are removed during mRNP remodeling by the action of the DEAD-box ATPase Dbp5 and its activating cofactor Gle1. The mechanism for remodeling specificity, by which certain RNA binding proteins in the mRNP are targeted for remodeling, has not been fully defined. In *S. cerevisiae*, the conserved NPC components Nup159 (human NUP214) and Nup42 (hCG1) are asymmetrically localized to the cytoplasmic face and both contain a phenylalanine-glycine (FG) repeat domain that binds mRNP transport receptors. In addition, Nup159 has a separate domain for Dbp5 docking, and Nup42 interacts with Gle1. We speculated that the Nup42 and Nup159 FG domains function to position the mRNPs for the terminal mRNP remodeling steps carried out by Dbp5. Indeed, deletion (Δ) of both the Nup42 and Nup159 FG domains resulted in a cold-sensitive poly(A)+ mRNA export defect. Furthermore, the *nup42 Δ FG nup159 Δ FG* mutant had synthetic lethal genetic interactions with *dbp5* and *gle1* mutants and a reduced capacity for mRNP remodeling during export. To further analyze the FG domain roles, we replaced the Nup159 or Nup42 FG domains with those from other Nups. These FG “swap” constructs differentially rescued the synthetic lethal *rat8-2 (dbp5) nup42 Δ FG nup159 Δ FG* triple mutant. Thus, only certain FG domains are functional at the NPC cytoplasmic face, providing key evidence that FG character and context play a direct role in FG domain function and mRNA export. Strikingly, fusing the Nup42 FG domain to the carboxy-terminus of Gle1 bypassed the need for the endogenous Nup42 FG domain, highlighting the importance of proximal positioning for these factors. We conclude that the Nup42 and Nup159 FG domains target specific proteins in the mRNP for remodeling by Dbp5 at the NPC. Our ongoing studies are further examining the molecular determinants for directional mRNA export through the NPC with an aim to determine the sequence of interactions between Nup159, Nup42, Gle1, Dbp5, and the mRNP.

P368**Board Number: B509****Roles of nuclear filamentous-actin in transcriptional regulation.**S. Yamazaki¹, K. Yamamoto¹, K. Sakata-Sogawa^{2,3}, M. Tokunaga^{2,3}, M. Harata¹;¹Graduate School of Agricultural Science, Tohoku University, Sendai, Miyagi, Japan, ²Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama, Kanagawa, Japan, ³Center for Integrative Medical Sciences, RIKEN, Yokohama, Kanagawa, Japan

In the cytoplasm, actin function is an integral component of the cytoskeleton, which plays crucial roles in a variety of cell functions. It has been revealed that actin exists also in the nucleus. However, the function and properties of nuclear actin remain elusive. Recently, we showed that actin tagged with EYFP fused with a nuclear localization signal (EYFP-NLS-actin) formed visible filamentous (F)-actin bundles in a small portion of cells, indicating the potential of actin to form filamentous architecture in the nucleus [1]. Moreover, we found that nuclear F-actin enhances general transcription. To obtain more detail about individual genes affected by nuclear actin, we applied microarray analysis using EYFP-NLS-actin expressing HeLa cells and showed that nuclear F-actin induces the expression of transcription factor genes including *OCT4* [2]. Together with a previous observation that nuclear F-actin activated *Oct4* in *Xenopus* oocytes [3], this observation suggests that this activation process is conserved in vertebrates and among cell types. To pursue mechanisms of transcription activation by nuclear F-actin, we analyzed the relationship between nuclear actin and β -catenin, which is the central transcription factor in the Wnt signaling pathway and has a role in *OCT4* transcription. Interestingly, nuclear accumulation of β -catenin was observed in HeLa cells expressing EYFP-NLS-actin, suggesting a role for nuclear F-actin in tethering β -catenin near chromatin. A luciferase reporter assay showed that the accumulation of F-actin in the nucleus enhances Wnt/ β -catenin signaling activity. These observations imply important roles of nuclear F-actin in transcriptional regulation.

References:

1. A. Kalendová, I. Kalasová, S. Yamazaki, L. Uličná, M. Harata and P. Hozák, *Histochem. Cell Biol.*, 142, 139-152 (2014).
2. S. Yamazaki, K. Yamamoto, M. Tokunaga, K. Sakata-Sogawa and M. Harata, *Biosci. Biotechnol. Biochem.*, in press (2014).
3. K. Miyamoto, V. Pasque, J. Jullien and JB. Gurdon, *Genes Dev.*, 25, 946-58 (2011).

P369**Board Number: B510****Nucleocytoplasmic relocation of actin in cell differentiation.**K. Yamamoto¹, S. Yamazaki¹, K. Sakata-Sogawa^{2,3}, M. Tokunaga^{2,3}, M. Harata¹;¹Graduate School of Agricultural Science, Tohoku University, Sendai, Miyagi, Japan, ²Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama, Kanagawa, Japan, ³Center for Integrative Medical Sciences, RIKEN, Yokohama, Kanagawa, Japan

Actin is involved in the regulation of multiple cytoplasmic functions, including cell motility, division and morphological changes. In addition, recent studies revealed that actin plays roles in epigenetic regulation. The nuclear function of actin is partly achieved by the inclusion of G-actin into chromatin remodeling complexes together with actin-related proteins (Arps). In addition, it is hypothesized that the formation of nuclear filamentous-actin (F-actin) affects functional nuclear organization. To analyze the involvement of nuclear actin in cell differentiation, we have investigated the human keratinocyte HaCaT cell line. Cell differentiation of HaCaT cells is induced with Ca^{2+} , and the progression of cell differentiation was observed by monitoring the expression level of Keratin10. When EYFP-actin or Lifeact, a probe which specifically binds to F-actin, was transfected into HaCaT cells, a transient increase of nuclear actin was observed according to the progression of cell differentiation. To induce an artificial decrease in nuclear actin, we applied Jasplakinolide, an inducer of actin polymerization. Jasplakinolide treatment reduces the G-actin pool in the cytoplasm through F-actin formation, and as a result, import of G-actin into the nucleus is reduced. The Jasplakinolide treatment repressed the differentiation of HaCaT cells, supporting the idea that accumulation of nuclear actin is required for cell differentiation. We hypothesized that one potential role for nuclear actin is in tethering β -catenin, a molecule central to Wnt signaling near chromatin via F-actin. Microscope observations showed that nuclear β -catenin is increased in HaCaT cells expressing EYFP-actin tagged with a nuclear localization signal and that nuclear F-actin is co-localized with β -catenin. These results imply that nuclear actin is involved in cell differentiation, at least, partly through affecting Wnt/ β -catenin signaling.

References:Y. Oma and M. Harata, *Nucleus* 2,38-46(2011).S. Yamazaki, K. Yamamoto, M. Tokunaga, K. Sakata-Sogawa and M. Harata, *Biosci. Biotechnol. Biochem.*, in press (2014).

Endocytic Trafficking 1

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Board Number: B512

Altered Clathrin Dependent and Independent Endocytosis in Lysosomal Storage Disease Cells and Therapeutic Rescue by Storage Attenuation.

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Endocytosis and endo-lysosomal trafficking of extracellular materials and plasmalemma components regulate a plethora of biological functions. Yet, whether disease disrupts these processes remains largely unexplored. An example is that of lysosomal storage diseases (LSDs), a group of 50 genetic conditions where lysosomal enzyme deficiency causes accumulation of undegraded substances, with potential to affect endocytosis. Using fluorescence microscopy, we examined fluid-phase and ligand endocytosis via clathrin, caveolae, and macropinocytic pathways. We compared wild-type fibroblasts to those of patients with type A Niemann-Pick (NPD), type C Niemann-Pick (NPC), Gaucher, and Fabry diseases, characterized by different protein deficiencies and accumulated substrates. First, using NPD we observed altered distribution of clathrin heavy-chain and caveolin-1 toward the perinuclear region (clathrin: 85% vs. 36% in wild-type, caveolin-1: 88% vs. 28% in wild-type), and poor co-localization of these markers with transferrin (17% vs. 63% co-localization in wild-type; 1 hr) and cholera toxin B (CTB; 8% vs. 76% co-localization in wild-type). Uptake of both ligands was significantly reduced: 76% and 71% reduction in transferrin- and CTB-positive endosomes, and 20% and 35% reduced uptake rate. Bulk pinocytosis, visualized using Texas Red dextran, was decreased by 40%, and the use of single or combination pharmacological inhibitors confirmed a decreased fluid-phase uptake via both clathrin and caveolar pathways. EGF-induced macropinocytosis was also affected (67% of wild-type). Imipramine induction of lysosomal storage in wild-type fibroblasts altered endocytosis similarly to genetic NPD, and pharmacological removal of storage in NPD cells restored uptake. This suggests that lysosomal storage causes reduced endocytosis. Indeed, examination of fibroblasts from other LSDs (NPC, Gaucher, and Fabry diseases) confirmed reduced uptake via clathrin dependent (77%, 88%, and 76% reduction) and independent (68%, 43%, and 51% reduction) pathways. As a result, endocytosis of a recombinant lysosomal enzyme (NPD acid sphingomyelinase) aimed at replacement therapy, which relies on the clathrin-dependent mannose-6-phosphate receptor, was 10-fold lower as compared to enzyme delivery via drug carriers targeted to ICAM-1, known to bypass classical endocytic routes. The uptake of free enzyme vs. carrier-coupled enzyme correlated well with the reduction of lysosomal storage (3.3- vs. 1.4-fold of wild-type), and the carriers led to recovery of endocytic function (71% recovery of clathrin-mediated uptake and 150% of caveolae-mediated uptake). Endocytic behavior can differ dramatically in healthy vs. pathological conditions and this knowledge can guide the design of therapeutic interventions.

P371**Board Number: B513****Characterizing Novel Sla1-Associated Mechanisms in Clathrin Mediated Endocytosis.**T.O. Tolsma¹, S.M. Di Pietro¹;¹Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO

Clathrin mediated endocytosis is an essential cellular process used by all eukaryotes. Its many roles include the uptake of extracellular nutrients, manipulation of plasma membrane composition, and regulation of signal transduction pathways. With such a high degree of necessity, it is understandable why various disorders have been linked to the mutation of proteins involved in clathrin mediated endocytosis, including hypercholesterolemia and Alzheimer's. The process begins with the initial recruitment of specialized adaptors and early endocytic proteins to the plasma membrane. Here they aggregate through the binding of membrane proteins, receptors, and/or lipids, to establish the site of endocytosis. Clathrin, the defining component of the process, is incapable of binding membrane receptors or lipids, and requires adaptor proteins for its sequential recruitment. Next, the plasma membrane will invaginate, and a clathrin lattice will form around the lipid surface until scission occurs, and a protein coated vesicle is released into the cytoplasm. While the basic process of clathrin mediated endocytosis has been well documented, little is known about the regulatory mechanisms that control clathrin recruitment and coat formation at the plasma membrane. Since clathrin not only acts as a scaffold for the recruitment and function of various endocytic proteins, but is also believed to be an important contributor of vesicle formation and structure, identifying mechanisms that contribute to its recruitment is necessary in generating a proper understanding of clathrin mediated endocytosis.

Previously, our lab established Sla1 as the first clathrin adaptor to contain a sterile alpha motif, or SAM domain, with a role in endocytosis. Oligomerization of Sla1 through its SAM domain resembles other SAM domain proteins, but the ability to regulate its own clathrin binding was a novel function of Sla1. Our current work demonstrates a role for Sla1 in clathrin recruitment and/or coat formation. This is based on fluorescent confocal microscopy imaging of Sla1-GFP and the RFP tagged clathrin heavy chain subunit, as well as immunoblotting for clathrin in membrane and cytosol fractions of cells with mutation to the clathrin binding domain of Sla1. Furthermore, imaging of Sla1-GFP with various RFP tagged endocytic protein markers indicated a role for Sla1-Clathrin binding in normal protein dynamics, and progression of late stage mechanisms in endocytosis. In these experiments the levels of RFP tagged proteins at endocytic sites was also significantly higher, possibly indicating a compensatory mechanism resulting from defective Sla1-Clathrin binding. The goal of this project is to further our understanding of the functional role Sla1-Clathrin binding plays in endocytosis.

P372**Board Number: B514****The role of ligand size and receptor engagement density on ceramide-dependent, clathrin- and caveolae-independent endocytosis via the CAM-mediated pathway.**D. Serrano¹, E. Paul², S. Muro^{2,3};¹Biological Sciences Graduate Program, University of Maryland, College Park, MD, ²Fischell Department of Bioengineering, University of Maryland, College Park, MD, ³Institute for Bioscience and Biotechnology Research, University of Maryland, College Park, MD

Eukaryotic cells display endocytic pathways associated with uptake of multivalent ligands that can vary in size and density of receptor engagement, relevant to both their biology and translational applications. Yet, the role of these parameters in endocytosis remains relatively unexplored. We have previously described the clathrin- and caveolae-independent cell adhesion molecule (CAM)-mediated pathway, through which cells internalize natural (pathogens) and artificial (drug carriers) particle-ligands 0.1-10 μm in diameter, allowing for the systematic study of these parameters. This pathway is induced by multivalent binding to intercellular adhesion molecule 1 (ICAM-1), which recruits Na⁺/H⁺ exchanger 1 (NHE1) and acid sphingomyelinase (ASM) to binding sites. Concerted NHE1/ASM activity renders ceramide production, responsible for the size flexibility of CAM-mediated endocytosis. Here, we examined this pathway as a model to explore the role of ligand size and receptor engagement density in endocytosis. We used polystyrene particles 0.2, 1, or 4.5 μm in diameter coated with varying densities of anti-ICAM to determine binding, uptake, and ceramide enrichment using endothelial cells and fluorescence microscopy. The larger the particle, the lower the number of particles bound per cell ($\approx 100, 25, 15$; 30 min). Yet, with time (24 h), total binding reached the same total cell-surface area occupancy ($\approx 400 \mu\text{m}^2$), coinciding with saturation of available ICAM-1 molecules. Initially, particles of 0.2 and 1 μm exhibited more efficient uptake than 4.5 μm particles (70% vs. 30% uptake; 30 min), but a similar total uptake was found at saturation (90%; 24 h). Micron-sized particles elicited higher ceramide enrichment than nanometer-sized counterparts (5- vs. 2-fold over background levels). Since larger particles internalized more slowly, this suggests that larger particles require higher ceramide production for uptake. Linear regression showed that ceramide generation correlated well with density of ICAM-1 engagement of these particles. Hence, receptor signaling rendering ceramide enrichment depends on the density of receptor engagement of each individual particle, regardless of the total number of particles bound per cell. Indeed, particles with the same size but different anti-ICAM densities showed that higher ICAM-1 engagement density, accompanied with higher ceramide enrichment, elicited more efficient uptake. Overall, this work suggests that ceramide enrichment, which can be modulated by changing receptor engagement density, affects endocytosis in a size-dependent manner. This information will be valuable in the design of particles for intracellular drug delivery and may also help elucidate the ceramide-dependent endocytic mechanisms of natural p

P373**Board Number: B515****Constitutive Endocytosis and Endocytic Recycling of the Sodium-dependent Vitamin C transporter 2 (SVCT2) are regulated by ascorbic acid in a Clathrin and Rab11-dependent manner.**A.D. Covarrubias-Pinto¹, E. Papic², P. Burgos³, M.A. Castro²;¹I. Bioquímica, Universidad Austral De Chile, Valdivia, Chile, ²Universidad Austral De Chile, Valdivia, Chile,³Department of Fisiology, Universidad Austral De Chile, Valdivia, Chile

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Ascorbic acid is an essential antioxidant molecule in the physiology of the brain and other organs. It is transported into the brain and neurons via the Sodium-dependent Vitamin C Transporter-2 (SVCT2). During synaptic activity ascorbic acid is released from glial reservoirs to extracellular space. Results from our laboratory have demonstrated that SVCT2 is continuously cycling to and from plasma membrane. An increase in extracellular ascorbic acid is able to induce an increase in SVCT2 localization at the plasma membrane. However the endocytic recycling mechanisms that regulate the surface expression of SVCT2 are still unknown. Methods and Results: Using HEK293T cells expressing endogenous SVCT2 we evaluated SVCT2 trafficking in presence of ascorbic acid. Immunofluorescence analyses and biotinylation assays showed an increase in surface levels of SVCT2 in cells exposed to extracellular ascorbic acid. No changes in total protein levels were observed. Inhibition of Clathrin-mediated endocytosis by K⁺ depletion showed an increase of SVCT2 levels at the cell surface. The expression of SVCT2 at the plasma membrane was increased in cells overexpressing a dominant negative Dynamin II (K44A). Dynamin is involved in Clathrin-dependent and independent endocytosis. Therefore, we evaluated surface expression of SVCT2 using Clathrin endocytosis inhibitors (cells overexpressing AP180 or dominant negative mutants of Eps15) which resulted in increased levels of SVCT2 at the plasma membrane. Finally, dominant negative versions of Rab5 or Rab11 abolished the ascorbic acid-induced increase of SVCT2 at the cell surface. Discussion: Our results revealed that the ascorbic acid-induced increase in plasma membrane SVCT2 is regulated by Clathrin and Rab11 dependent mechanisms. FONDECYT1110571 (MC), FONDECYT 1130929 (PVB), CONICYT Scholarship (ACP) and DID- UACH.

P374**Board Number: B516****Epsin deficiency impairs endocytosis by stalling the actin-dependent invagination of endocytic clathrin coated pits.**M. Messa¹, R. Fernandez-Busnadiego², E.W. Sun³, H. Chen⁴, H. Czaplak¹, K. Wrasman⁵, Y. Wu^{3,6,7}, G. Ko³, T. Ross⁸, B. Wendland⁵, P. De Camilli²;¹Cell Biology, Yale University School of Medicine/HHMI, New Haven, CT, ²Department of Cell Biology, Howard Hughes Medical Institute, Program in Cellular Neuroscience, Neurodegeneration and Repair, Yale University School of Medicine, New Haven, CT, ³Department of Cell Biology, Yale University, New Haven, CT, ⁴Oklahoma Med Res Fund, Oklahoma City, OK, ⁵Department of Biology, Johns Hopkins University, Baltimore, MD, ⁶department of cell biology, 1Howard Hughes Medical Institute, New Haven, CT, ⁷CNNR program, Yale University, New Haven, CT, ⁸Division of Hematology-Oncology, University of Texas Southwestern Medical Center, Dallas, TX

Epsin is an evolutionarily conserved endocytic clathrin adaptor, which is encoded by three different genes (*EPN1*, *EPN2* and *EPN3*) in mammals. Both housekeeping roles in clathrin-mediated endocytosis and a specific role in the clathrin-dependent internalization of ubiquitinated cargos have been attributed to epsin. However, its most critical function(s) in metazoan cells remain(s) elusive. To elucidate such function(s), we generated embryonic fibroblasts from conditional epsin triple knock out (TKO) mice. These cells displayed a dramatic increase in cell size and in the number and/or size of nuclei, demonstrating a role of epsin in cell division, as previously reported for other endocytic factors including clathrin. Additionally, a global defect in clathrin-mediated endocytosis with the accumulation of shallow and U-shaped pits was observed in TKO cells, supporting a role of epsin in clathrin coat invagination. These changes correlated with the accumulation of actin foci at the cell surface, typically in close proximity of arrested coated pits. A similar actin defect was previously observed in Hip1R knockdown cells. Accordingly, we found that Hip1R, a factor known to directly link the clathrin coat to actin, was no longer recruited to endocytic clathrin coated pits both in intact cells and in a cell free system. We also found that the ENTH domain of epsin binds Hip1R, while its unfolded "tail" binds actin directly. These findings, which point to a role of epsin in providing a link between endocytic clathrin coats and actin leading to deep invagination, reconcile observations on epsin function previously made in yeast and dictyostelium, with the function of epsin in metazoan cells. Finally, we report here a low affinity interaction, but potentiated by the presence of PI(4,5)P₂, between the SNARE motif of synaptobrevin2/VAMP2 and the ENTH domain of epsin. Such interaction, which is conserved from yeast to mammals, may help couple clathrin coat nucleation to incorporation of a SNARE in the nascent bud. Collectively, our results demonstrate a housekeeping role of epsin in clathrin-mediated endocytosis, in addition to cargo specific functions as suggested by previous studies. A key function of this protein is to help generate the force that leads to the invagination of clathrin coated pits as a premise to their fission.

P375**Board Number: B517****Investigating new protein components of the endocytic machinery in *Saccharomyces cerevisiae*.**K. Farrell¹, S.M. Di Pietro¹;¹Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO

Clathrin-mediated endocytosis is an important process which allows cells to control membrane lipid and protein content, signaling processes, and uptake of nutrients among other functions. The around 60 known proteins that compose the endocytic machinery in *Saccharomyces cerevisiae*, or budding yeast, have been identified and investigated by taking advantage of the ease of genetic manipulations and two-color fluorescent microscopy in this model organism. Clathrin-mediated endocytosis is highly conserved between yeast and mammals in terms of both protein content and precise timing of protein arrival. First in the endocytic process, there is an immobile phase in which clathrin and other coat components concentrate at endocytic sites. Second, another wave of proteins, such as adaptor protein Sla1, assembles about 20 seconds before actin polymerization. Third, a fast mobile stage of endocytosis occurs coinciding with actin polymerization and culminates with vesicle scission. Fourth, most coat proteins disassemble from the internalized vesicle. Despite the knowledge of so many endocytic proteins, gaps still remain in the complete understanding of the endocytic process. We attempt to fill some of these gaps with a screen of the yeast GFP library for novel endocytic-related proteins. The screen was performed with still and time lapse images using confocal microscopy. We identified proteins colocalizing with RFP-tagged Sla1, an adaptor protein present at all sites of clathrin-mediated endocytosis. Use of the GFP library will reveal proteins that have not previously been identified in knockout screenings due to viability issues or do not show an endocytic defect due to redundancy of protein function. Our screen revealed eight potential novel endocytic proteins that are currently uncharacterized. Ubx3, an unstudied protein, was selected first for further investigation based on high degree of colocalization with Sla1. Ubx3 contains a UBX domain that is structurally similar to ubiquitin, which plays multiple roles in the endocytic process. Ubx3 shows fluorescent patch dynamics similar to an endocytic coat protein. Ubx3 binds clathrin and is dependent on clathrin for patch lifetime. A knockout strain of Ubx3 displays a small but significant reduction in bulk endocytosis by Lucifer Yellow and Mup1 assays. The Ubx3 knockout also shows a significant increase in lifetime of early endocytic protein Ede1, possibly accounting for the defect in internalization. Thus, we have identified a novel component of endocytic machinery by screening the yeast GFP library.

P376**Board Number: B518****The α -arrestin ARRDC3 regulates ALIX-mediated GPCR lysosomal sorting.**M. Does¹, H. Lin¹, H. Buck¹, F. Mendez¹, J. Trejo¹;¹Pharmacology, University of California, San Diego, La Jolla, CA

G protein-coupled receptors (GPCRs) represent the largest family of transmembrane signaling receptors expressed in mammals, and lysosomal degradation of GPCRs serves to attenuate receptor signaling and desensitize cells to persistent activation. GPCR lysosomal sorting defects cause aberrant cellular responses that contribute to many diseases, including chronic inflammation and cancer metastasis. Given the diversity of GPCRs, multiple pathways exist to sort different receptors into lysosomal compartments. We have discovered a subset of GPCRs that are targeted to lysosomes by an intracellular loop 2 localized YPX_nL (where X is any amino acid) motif that are bound by the ESCRT adaptor protein ALIX. This class of GPCRs includes the purinergic receptor P2Y₁ and protease-activated receptor-1 (PAR1), the GPCR for thrombin. To investigate how ALIX-mediated GPCR lysosomal sorting is regulated, we assayed ALIX-interacting proteins for their role in YPX_nL GPCR degradation. Here, we demonstrate that the human alpha-arrestin ARRDC3 regulates ALIX-mediated lysosomal sorting of PAR1 by modulating ALIX endosomal sorting activity. ARRDC3 interacts with PAR1 and colocalizes with both PAR1 and ALIX at endosomes. ARRDC3 is required for PAR1 degradation, but not for the degradation of ubiquitinated signaling receptors. Depletion of ARRDC3 inhibits ALIX interaction with PAR1 and CHMP4, a subunit of ESCRT-III, which facilitates cargo sorting into late endosomes/lysosomes. ARRDC3 is also required for ALIX ubiquitination, a possible mechanism for regulating ALIX function at late endosomes. ARRDC3 interacts with multiple members of the Nedd4 E3 ubiquitin ligase family and may serve as an adaptor for ALIX ubiquitination. An siRNA screen of all nine human Nedd4 ligases revealed a specific function for WWP2 in the degradation of PAR1. This study reveals a novel role for alpha-arrestins and the E3 ligase WWP2 in the endosomal trafficking of non-ubiquitinated GPCRs through the regulation of the adaptor protein ALIX during lysosomal sorting.

P377**Board Number: B519****Analysis of the endocytic recycling compartment with super-resolution microscopy.**S. Xie¹, N. . Naslavsky¹, S. Caplan¹;¹Univ Nebraska Med Ctr, Omaha, NE

Endocytic recycling and endocytosis are coordinated to regulate the equilibrium of protein and lipids on the plasma membrane. Recycling occurs either directly (fast recycling), or via an organelle known as the endocytic recycling compartment (ERC). The composition of the ERC is poorly understood and is typically described as a series of tubular-vesicular membrane-bound organelles localized to the peri-nuclear region of the cell. Prominent ERC proteins include the small GTPases Rab11 and Rab8, Molecule

Interacting with Cas1-Like protein 1 (MICAL-L1), motor proteins such as Myosin Vb and KIF13a, and other regulators of recycling endosomes such as the C-terminal Eps 15 Homology Domain (EHD) proteins. Our understanding of endocytic recycling is influenced significantly by studies on transferrin, a protein internalized through clathrin-dependent endocytosis. After internalization, transferrin is sorted to Rab11-enriched recycling endosomes at the ERC and recycled to the plasma membrane in a Myosin Vb-dependent manner. However, the sorting and recycling of clathrin-independent cargos such as Major Histocompatibility Complex Class I (MHC I) and the glycosylphosphatidylinositol-anchored protein, CD59 remains ill-defined. We show that transferrin highly colocalizes with over-expressed Myosin Vb tail in the peri-nuclear area and its recycling is delayed. Using Structured Illumination Microscopy (SIM), we discovered that MHC I, CD59 and CD98 are unaffected by the overexpression of GFP-Myosin Vb tail. Moreover, although transferrin and CD59 appear to be sorted to the same peri-nuclear region, SIM analysis shows that they reside on distinct vesicles within the ERC, in close proximity with one another. Further studies on the molecular architecture and sorting of recycling endosomes will be performed with SIM- and Photoactivated Localization Microscopy (PALM)-based analysis of the key players of the recycling system. These include Rab11, MICAL-L1, EHD1, Myosin Vb and KIF13a. These data, combined with the kinetic analysis of the recycling of different cargos, will provide an in-depth understanding of the ERC, and novel insight into ERC biogenesis and function.

P378

Board Number: B520

Macropinocytosis as a model for tracking clathrin-independent cargo sorting in space and time.

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After internalization by clathrin-independent endocytosis (CIE), incoming endosomes fuse to form a sorting endosome where cargo proteins are sorted for targeting to lysosomes for degradation or for recycling back to the PM. Our lab has identified CIE cargo proteins (CD44, CD98, and CD147) that contain cytosolic acidic motifs, allowing them to interact with the Hook1 adaptor protein and be rapidly recycled back to the plasma membrane via recycling tubules. Other CIE cargo proteins such as MHC Class I (MHCI) and the GPI-anchored protein CD59 remain within the sorting endosome as it matures and can then be directed into MVBs and late endosomes for degradation, or delivered back to the plasma membrane via the juxtannuclear endocytic recycling compartment. In HeLa cells, sorting of these two types of cargo proteins can be detected by colocalization of the proteins with EEA1, the kinetics of protein entry into recycling tubules, or the reappearance of endocytosed proteins back at the plasma membrane. The differential sorting of CD44, CD98 and CD147 allows these proteins to recycle to the PM directly and avoid degradation in lysosomes, making them long-lived proteins. In order to visualize cargo sorting in real-time we turned to a stimulated form of CIE, macropinocytosis, which enables cargo sorting to be captured on individual macropinosomes as they mature. The HT1080 fibrosarcoma cell line exhibits constitutive macropinocytosis due to expression of an activated form of N-Ras. We found that CD98 and

CD59 enter cells on actin and PI(3,4,5)P₃ coated macropinosomes newly formed in the lamellar region of HT1080s. After formation, these macropinosomes are pushed up against an actin barrier at the base of the lamella. Loss of actin from both the macropinosome and the base of the lamella is observed, followed by a deformation of the macropinosome as it squeezes through the barrier. Subsequent acquisition of Rab5 on the macropinosome is quickly followed by fusion with other macropinosomes and transient association of the Rab5 adaptor protein APPL1. This allows the sorting of CD98 into puncta and small tubules that emanate from the macropinosome, leaving CD59 behind. Thus, discrete cargo sorting events can be visualized by live cell microscopy in HT1080 cells, and are often captured on Rab5-positive endosomes containing either APPL1 or Hook1 proteins. This HT1080 model system compliments our current understanding of CIE protein sorting in HeLa cells, and uniquely augments our ability to visualize and investigate the mechanisms involved in endosomal cargo sorting.

P379

Board Number: B521

Membrane raft association is a determinant of plasma membrane localization.

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The organization of metazoan membranes into functional domains is a key feature of their physiology. The lipid raft hypothesis proposes lateral domains driven by preferential interactions between sterols, sphingolipids, and specific proteins as a central mechanism for the regulation of membrane structure and function; however, experimental limitations in defining raft composition and properties have prevented unequivocal demonstration of their function. In this study we establish a functional relationship between raft association and subcellular protein sorting. To quantify raft association in intact plasma membranes without detergent perturbation, we isolated Giant Plasma Membrane Vesicles (GPMVs) from cultured mast cells. These GPMVs separate into coexisting raft and non-raft phases, as defined by physical properties and lipid/protein compositions. This system provides an ideal model to microscopically analyze the structural determinants and functional consequences of protein association with membrane domains. By systematic mutation of the transmembrane and juxtamembrane domains of an integral single pass plasma membrane protein, linker for activation of T-cells (LAT), we generated a panel of variants possessing a range of raft affinities. These mutations revealed palmitoylation, transmembrane domain length, and transmembrane sequence to be critical and independent determinants of membrane raft association. Having identified the structural features determining raft association, we used our panel of mutants to establish a quantitative, functional relationship between raft association and subcellular protein sorting. We observed that plasma membrane (PM) localization was strictly dependent on raft partitioning across the entire panel of unrelated mutants, suggesting that raft association is necessary and sufficient for PM sorting of LAT. The specific defect in PM sorting was a failure to recycle non-raft variants from early endosomes, with abrogation of raft partitioning leading to mistargeting to late endosomes/lysosomes and subsequent degradation. Finally, we confirmed that

these observations are not specific to LAT, but are general to at least the three other unrelated single-pass transmembrane proteins. These findings identify structural determinants of raft association and validate lipid-driven domain formation as a mechanism for protein sorting in the endosomal system.

P380

Board Number: B522

The role of the ubiquitin E2 conjugating enzyme UbcH5c on trafficking of the G protein-coupled receptor CXCR4.

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G protein coupled receptors (GPCRs) belong to a superfamily of signaling receptors that mediate the cellular responses of most hormones and neurotransmitters. Dysregulated GPCR signaling is associated with several pathologies and GPCRs are often targeted clinically to treat a variety of diseases. The molecular mechanisms regulating GPCR signaling are complex and occur at multiple steps of the signal transduction cascade. In particular, direct modification of GPCRs by post-translational modifications such as phosphorylation and ubiquitination help to ensure that signaling is of the appropriate magnitude and duration. One function of these modifications is to promote receptor endocytosis and lysosomal targeting leading to GPCR degradation and long-term attenuation of signaling. For example, agonist-dependent ubiquitination of the chemokine receptor CXCR4, a prototypical GPCR, leads to its lysosomal sorting and degradation. However, the mechanisms regulating CXCR4 lysosomal sorting remain poorly understood. CXCR4 is targeted to lysosomes via the endosomal sorting complex required for transport (ESCRT) pathway. We have recently shown that ubiquitination of ESCRTs is important in regulating CXCR4 lysosomal degradation. In particular, the ubiquitination status of ESCRT-0 subunits HRS and STAM1 is regulated by two E3 ubiquitin ligases: atrophin-1 interacting protein 4 (AIP4) and deltex-3L (DTX3L). DTX3L interacts with AIP4 *in vitro* and inhibits the auto-ligase activity of AIP4. Our data suggest that ubiquitination of ESCRT-0 inhibits its sorting activity, which regulates the amount of CXCR4 targeted for lysosomal degradation. However, the precise interplay by which AIP4 and DTX3L operate to regulate ubiquitination of ESCRT-0 remains poorly understood. To explore this further, here we focused on the ubiquitin E2 conjugating enzymes that operate with DTX3L and AIP4 in cellular ubiquitination assays. AIP4 has been shown to operate with E2 enzymes UbcH5c and UbcH7, while DTX3L appears to selectively function with UbcH5c in ubiquitination reactions. Using several biochemical and immunochemical techniques including fixed cell confocal immunofluorescence microscopy coupled with *in vitro* ubiquitination assays, we sought to determine the role of the E2 enzymes in CXCR4 trafficking. We show that depletion of UbcH5c by siRNA in HeLa cells, but not its closely related isoforms UbcH5a or UbcH5b, attenuates agonist promoted degradation of CXCR4. UbcH5c depletion prevents CXCR4 trafficking from early endosomes to lysosomes, thereby, preventing CXCR4 from being degraded in the lysosome. In addition, endogenous UbcH5c colocalizes with DTX3L on the surface of early endosomes. Based on these data we hypothesize that UbcH5c operates with AIP4 and DTX3L to regulate the extent

of CXCR4 sorting into the degradative pathway. Thus, we have defined a novel role for UbcH5c in GPCR endosomal sorting.

P381

Board Number: B523

Cortical microtubule capture machinery contributes to the yeast clathrin-independent endocytic pathway.

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Although most cell types use both clathrin-mediated (CME) and clathrin-independent (CIE) endocytic pathways, yeast have been thought to rely solely upon CME for internalization of plasma membrane cargo. We recently uncovered a second endocytic pathway in yeast that acts independently of clathrin and CME machinery, and instead relies on the small GTPase Rho1 and its effector, the formin Bni1. To date, all proteins with an identified role in the CIE pathway do not localize to the cortical actin patches that are known sites of CME, suggesting that CME and CIE occur at distinct sites. However, the mechanism by which CIE occurs in yeast is currently unclear. Here, we show that Bni1 and Spa2, a polarisome protein involved in CIE, form dynamic, short-lived patches at the cell cortex that are distinct from CME machinery proteins such as the early-coat factor Ede1 and the actin module component Abp1. Notably, several proteins implicated in CIE, including Bni1 and the polarity protein Bud6, are also linked to cortical capture of cytoplasmic microtubules. Depolymerization of microtubules with nocodazole and deletion of additional proteins involved in cortical microtubule capture inhibited the CIE pathway. Furthermore, mutation of the type V myosin Myo2, which directs vesicle, organelle and microtubule transport along actin cables, also reduced the efficiency of cargo internalization via the CIE pathway. Taken together, these data indicate that CME and CIE are spatially and mechanistically distinct, and suggest an unexpected role for the microtubule cytoskeleton in the yeast CIE pathway.

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Board Number: B524

Microtubule motors drive membrane tubulation in clathrin-independent endocytosis.

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How the plasma membrane is bent to accommodate clathrin-independent endocytosis is poorly understood. Recent studies suggest the exogenous clathrin independent cargo molecules Shiga and cholera toxin induce the negative membrane curvature required for endocytic uptake by binding and cross-linking multiple copies of their glycosphingolipid receptors on the plasma membrane. But it remains unclear if toxin-induced sphingolipid crosslinking provides sufficient mechanical force for deforming the plasma membrane, or if host cell factors also contribute to this process. To test this, we imaged the uptake of cholera toxin B-subunit into surface-attached tubular invaginations in live cells. We found that a cholera toxin mutant that binds to only one glycosphingolipid receptor accumulates in tubules, and that toxin binding is entirely dispensable for membrane tubulations to form. Unexpectedly, the driving force for tubule extension was found to be supplied by the combination of microtubules, dynein, and dynactin, thus defining a novel mechanism for generation of membrane curvature during endocytic uptake at the plasma membrane.

P383

Board Number: B525

5-HT6 receptor is negatively regulated by sorting nexin 14.

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The 5-HT6 receptor (5-HT6R) plays roles in cognition, anxiety, and learning and memory disorders, yet new details concerning its regulation remain poorly understood. In this study, we found that 5-HT6R directly interacts with SNX14 and this interaction dramatically increases internalization and degradation of 5-HT6R. Knockdown of endogenous SNX14 has the opposite effect. SNX14 is highly expressed in the brain and contains a putative regulator of G-protein signaling (RGS) domain. Although its RGS domain is found to be non-functional as a GTPase activator for Gas, we found that it specifically binds and sequesters Gas, thus inhibiting downstream cAMP production. We further found that PKA mediated phosphorylation of SNX14 inhibited its binding to Gas and diverted SNX14 from Gas binding to 5-HT6R binding, thus facilitating the endocytic degradation of the receptor. Thus, our results suggest that SNX14 is a dual endogenous negative regulator in 5-HT6R-mediated signaling pathway, modulating both signaling and trafficking of 5-HT6R.

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Board Number: B526

COPI sorts ubiquitinated membrane proteins at early endosome.

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Coat proteins facilitate vesicle-mediated protein transport by selectively concentrating macromolecules into specialized membrane patches and then deforming these patches into small coated vesicles. In

budding yeast, the SNARE protein Snc1 recycles from early endosomes (EE) back to the trans-Golgi network (TGN) in a pathway that requires an ATP-powered phospholipid flippase (Drs2) and an F-box protein (Rcy1), but no clearly defined vesicle coat. Rcy1 has been implicated in ubiquitination of Snc1 (Ub-Snc1) to form a sorting signal for retrieval. To test if Snc1 uses an ubiquitin sorting signal, we fused the catalytic domain of a deubiquitinating peptidase (DUb) onto GFP-Snc1. EE to TGN transport of GFP-Snc1 was disrupted by DUb fusion to GFP-Snc1 while fusion with a catalytic inactive Dub had no effect. The sorting nexin 4 (Snx4) complex could potentially serve as a coat in this EE to TGN route; however, our results imply that Snx4 acts at a later endosome in a parallel pathway for recycling Snc1 and acts to prevent Ub-Snc1 entry into the multivesicular body pathway. COPI mutants perturb Snc1 EE to TGN transport although it is not known if COPI acts directly at the EE or if this is a secondary effect of the early secretory pathway defects. To test if COPI directly binds ubiquitinated cargo, we fused DUb and catalytic inactive DUb onto Sec27 (beta prime-COP) and Cop1 (alpha-COP). Sec27-DUb disrupted GFP-Snc1 trafficking while Sec27 fused with catalytic inactive DUb was without effect. Both Cop1-DUb and Cop1 fused with catalytic inactive DUb caused a partial GFP-Snc1 trafficking defect suggesting a nonspecific influence on alpha-COP function. Sec27-DUb does not appear to perturb COPI function in Golgi to ER transport. Structural alignment of the beta-propeller domain of Sec27 with Doa1 suggests an ubiquitin binding motif is present in this cargo-binding domain of Sec27. These results suggest that beta-COP directly selects ubiquitinated membrane protein at the EE for retrograde transport to the TGN.

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Board Number: B527

Apical-basal polarity reversal in epithelial cells by PtdIns (3, 4, 5) P3-containing gene delivery vectors: enhanced entry of polyplexes into epithelial cells.

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Polarized epithelial cells have a plasma membrane which is separated by tight junctions into two domains, apical and basolateral, with distinct protein and lipid compositions. The polarized architecture of epithelium provides a barrier to the invasion of exogenous pathogens *in vivo*. Likewise, epithelium represents a barrier to therapeutic drug and gene vectors. For efficient drug and gene delivery it is a challenge to efficiently deliver the therapeutic to epithelial barriers without damaging the integrity of the epithelial monolayer.

Apical addition of exogenous phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P₃; PIP₃), which normally localizes exclusively basolaterally, can locally transform the apical membrane into basolateral, as exemplified by the recruitment of basolateral receptors.

Here, we investigate if apical-basal polarity reversal enhances the uptake of gene vectors at the apical membrane of kidney epithelial cells.

Polyethylenimine (PEI) was used to complex PIP3 and/or DNA (coding for GFP), forming PEI/DNA/PIP3 terplexes and PEI/DNA complexes. Fluorescently labeled terplexes and complexes were added to polarized kidney epithelial cells, and binding/uptake of the gene vectors was measured using fluorescence microscopy/FACS analysis. At t=1hr, PEI/DNA/PIP₃ terplexes colocalize with the PIP3 sensor GFP-PH-Akt, transiently expressed in MDCK cells. In addition, basolateral receptors (β -integrin, syndecan-1, and transferrin receptor) are found to colocalize with terplexes at the apical membrane. At t=72 hrs, the cellular uptake of PEI/DNA/PIP3 is two-fold higher than that of PEI/DNA, although GFP expression is lower (0.80 \pm 0.29% for PEI/DNA/PIP₃; 2.58 \pm 2.11% for PEI/DNA). This can be explained by the entrapment of terplexes within cellular organelles (endosomes, lysosomes, mitochondria), which was visualized by immunostaining for the different intracellular organelles, and by transmission electron microscopic (TEM) observation. By the aid of photosensitizer TPPS_{2a} terplexes are released from endo/lysosomes as shown by the high nuclear accumulation of fluorescently labeled oligonucleotides in 77.5 \pm 4.7% of the cells. DNA was also seen to dissociate from endo/lysosomes upon TPPS_{2a} treatment, although this did not result in improved GFP expression.

We conclude that the apical addition of PIP3-containing gene vectors to polarized epithelial cells can recruit basolateral receptors to the apical surface, improving the uptake of the vectors by the cells. Successful release of genetic cargo from endosomal compartments can be induced by subsequent treatment with a photosensitizer. PEI/DNA/PIP₃ terplexes could be a promising delivery vector to epithelial barriers *in vivo*.

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Board Number: B528

A New Mode of Ligand-Dependent Notch Signaling.

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How binding of DSL receptor ligands to Notch leads to activation by proteases remains a matter of intense investigation. Current models must grapple with a cornucopia of experimental observations. First, Notch exists on the cell surface as a stable heterodimer because of the S1 cleavage. Second, Notch's LNR repeats must be destabilized to allow proteases access to the activating S2 cleavage site. Third, upon S3 cleavage, Notch's intracellular domain (NICD) gains access to the nucleus. Fourth, endocytosis of ligand in the ligand cell is indispensable for proper Notch function. Lastly, preferential transfer of Notch's extracellular domain (NECD) into the ligand cell (trans-endocytosis) correlates with induction of Notch signaling in the Notch cell. The 'pulling model' combines these observations into a sequence of events; endocytosis of the DSL receptor creates a force that either removes the NECD or deforms the LNR region enough to allow protease cleavage. To study this process in more detail, we designed a suite of new cell culture based assays that allow quantitative localization studies of the extra and intra cellular domain of Notch1 during interaction with its ligand Dll1. We provide direct evidence that the entire Notch1 receptor is trans-endocytosed into the Dll1 cell, where the separation of the

NECD and NICD occurs in novel endosomal structures. Further, we show that the remaining NICD is trafficked back to the Notch cell, in a process similar to exosome secretion, where subsequent proteolytic processing occurs. Lastly, we show that this activated Notch is able to transfer to a third 'recipient' cell. We believe that this new mode of Notch signaling provides deep insights into the mechanism of Notch signaling and offers a basis for studying Notch signaling in pattern formation beyond just two cells.

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Board Number: B529

Roles of Cerebral Cavernous Malformation 3 (CCM3) and Germinal Center Kinase III (GCKIII) in recycling endocytosis during tracheal tubulogenesis in *Drosophila*.

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Tubes of differing cellular architecture connect into networks. In the *Drosophila* tracheal system, two tube types connect within single cells (terminal cells); however, the genes that mediate this interconnection are unknown. We characterize two genes that are essential for this process: *lotus*, required for maintaining a connection between the tubes, and *wheezy*, required to prevent local tube dilation. We find that *lotus* encodes N-ethylmaleimide sensitive factor 2 (NSF2), whereas *wheezy* encodes Germinal center kinase III (GCKIII). GCKIIIs are effectors of Cerebral cavernous malformation 3 (CCM3), a protein mutated in vascular disease. Depletion of *Ccm3* by RNA interference phenocopies *wheezy*; thus, CCM3 and GCKIII, which prevent capillary dilation in humans, prevent tube dilation in *Drosophila* trachea. Ectopic junctional and apical proteins are present in *wheezy* terminal cells, and we show that tube dilation is suppressed by reduction of NSF2, of the apical determinant *Crumbs*, or of septate junction protein *Varicose*. More interestingly, we find that Rab11 accumulates in dilation area in *wheezy* terminal cell mutant and expression of Rab11 dominant negative form can highly suppress *wheezy* dilation phenotype, which suggests a role of GckIII in regulating recycling endocytosis to prevent tube dilations.

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Board Number: B530

Characterization of the mammalian Mon2/Ysl2.

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Mon2/Ysl2, which shares significant homology with Sec7 family Arf guanine nucleotide exchange factors, is poorly characterized in mammalian cells. Mon2 has been proposed as a guanine nucleotide exchange factor for a member of Arf family small GTPases—Arl1. Here, we present the first in depth characterization of mammalian Mon2. We found that Mon2 localized to trans-Golgi network which was

dependent on both its N and C termini. The depletion of Mon2 did not affect the Golgi localized or cellular active form of Arl1. Furthermore, our in vitro assay demonstrated that recombinant Mon2 did not promote guanine nucleotide exchange of Arl1. Therefore, our results suggest that Mon2 could be neither necessary nor sufficient for the guanine nucleotide exchange of Arl1. We demonstrated that Mon2 was involved in endosome-to-Golgi trafficking as its depletion accelerated the delivery of furin and CI-M6PR to Golgi after endocytosis.

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Board Number: B531

Superresolution imaging of endocytosis in yeast.

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Clathrin-mediated endocytosis is a highly intricate cellular process, which in yeast involves the ordered recruitment and disassembly of around 60 proteins. Diffraction-limited live-cell microscopy has led to tremendous insight into composition and dynamics of the endocytic machinery. Electron microscopy on the other hand offers nanometer resolution, but lacks molecular specificity. Thus, the structural organization of most endocytic proteins in situ is largely unknown.

We employ localization microscopy (PALM/STORM) to study endocytic structures in *Saccharomyces cerevisiae*. This method is ideal to study static structures, which is why cells are typically fixed during sample preparation, leading to the loss of temporal resolution. This is mirrored in considerable heterogeneity among the imaged endocytic sites, which complicates their interpretation. By blocking actin polymerization using latrunculin A, we arrest the endocytic sites in comparable states, allowing us to validate previously observed structures. Our current efforts focus on the coat and actin assembly preceding scission. Here, we were able to reveal subdiffraction features of the endocytic coat protein Sla1, which is involved in the regulation of actin polymerization. We show that Sla1 assembles in a ring shape independent of membrane curvature. We also visualized Abp1, which binds throughout polymerized actin at endocytic sites. By imaging a high number of endocytic sites, we attempt to describe the dynamic organization of the endocytic actin structures. Extending the approach by automated dual-color imaging, we are pursuing to obtain a comprehensive structural picture of the endocytic machinery in yeast.

Endosomes, Lysosomes, and Lysosome–Related Organelles 1

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Board Number: B532

Lysosomal adaptor proteins in health and disease.

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Lysosomes are ubiquitous membrane-bound intracellular organelles with an acidic interior, central for the degradation and recycling of macromolecules delivered by endocytosis, phagocytosis, and autophagy. The lysosome, alongside the proteasome, acts as a major degradative route for the cell. In particular, it is especially adapted for the removal of large structures that the proteasome cannot process. In particular, toxic protein aggregates, damaged organelles, such as ROS-producing mitochondria, intracellular pathogens and membranes as well as acting as a source of recycled nutrients such as amino acids and lipids. It is therefore no surprise that the lysosome and lysosomal pathways contribute, both positively and negatively, to the progression of multiple diseases including cancer. We are interested in disrupting lysosome function in cancer, with emphasis on protein complexes that maintain lysosome integrity and function. We have recently identified a PLEKHM1 as a scaffold protein that couples Rab7-HOPS complex with biogenesis of lysosome and regulation of contact points with other membrane including autophagosomes. Through the interaction with Rab7-HOPS, PLEKHM1 coordinates autophagosome maturation and is also a target for the intracellular Gram-negative bacteria Salmonella. We will present data on how this complex is coordinated and how Salmonella manipulate the Lysosomal compartment through PLEKHM1-Rab7-HOPS complex. We present new data on how these processes are regulated through post-translational modifications giving us new insights into how these adaptor complexes are regulated.

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Board Number: B533

Depletion of Beclin-1 increases the delivery of cathepsin D and EGF receptor to lysosomes.

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Introduction: Beclin-1 is an ubiquitous protein that has a central role in autophagy. Biochemically, Beclin-1 interacts with either Vps34/PtdIns3KC3 or UVRAG forming two stable complexes referred as Beclin-1 Complex 1 and 2, respectively. These two complexes regulate the formation of PI3P a crucial phospholipid in autophagosome formation. Recent observations indicate that Beclin-1 could also play autophagy-independent roles, such as in endocytosis and protein sorting, however to date it is unclear

the contribution of Beclin-1 in these processes. The aim of this study was to investigate the role of Beclin-1 in the delivery of cargoes to lysosomes. **Material and Methods:** To generate a stable cell line depleted in Beclin-1, H4 neuroglioma cells were infected with lentiviral particles encoding a specific shRNA to Beclin-1. These cells were used to analyze the maturation of cathepsin-D, a lysosomal aspartic protease, and the degradation of the epidermal growth factor receptor (EGFR), in response to its ligand. **Results:** We observed that depletion of Beclin-1 caused a significant increase in the levels of mature cathepsin-D, and in the rate of ligand-induced EGFR degradation. Consistent with this phenotype, we observed that depletion of Beclin-1 causes a significant increase in various adaptor proteins involved in the delivery of cargoes to lysosomes. Importantly, western blot analysis revealed that Beclin-1 depletion causes a significant decrease in UVRAG protein levels, indicating a destabilization of the Beclin-1 Complex-2. **Discussion:** Our results suggest a non-autophagic role for Beclin-1, probably connected to Beclin-1 Complex-2 that seems to operate as a negative regulator in the delivery of cargoes to lysosomes. Supported by FONDECYT 1130929, Beca CONICYT 21110746 and DID-UACH.

P392

Board Number: B534

Hepatitis B virus secretion is regulated through the activation of endocytic and autophagic compartments via Rab7 stimulation.

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The cellular mechanisms by which hepatitis B virus (HBV) is assembled and exported are largely undefined. Recently, it has been suggested that these steps require the multivesicular body (MVB) and the autophagic machinery. However, the mechanisms by which HBV might regulate these compartments are unclear. In this study we have found that by activating Rab7, HBV alters its own secretion by inducing dramatic changes in the morphology of MVB and autophagic compartments. These changes are characterized by the formation of numerous tubules that are dependent upon the increase in Rab7 activity observed in the HBV-expressing HepG2.2.15 cells compared to HepG2 cells. Interestingly, a transfection-based expression of the five individual viral proteins indicated that the precore protein, which is a precursor of HBeAg, was largely responsible for the increased Rab7 activity. Finally, siRNA-mediated depletion of Rab7 significantly increased the secretion of virions, suggesting that reduced delivery of the virus to the lysosome facilitates viral secretion. These novel findings provide evidence indicating that HBV can regulate its own secretion through an activation of the endo-lysosomal and autophagic pathways via Rab7 activation.

P393**Board Number: B535****The vacuole/lysosome is required for cell-cycle progression.**Y. Jin¹, L.S. Weisman¹;¹Life Sciences Institute, University of Michigan, Ann Arbor, MI

During the cell-cycle, cytoplasmic organelles are distributed to the new daughter cells. For example, in *S. cerevisiae*, there is active organelle transport from the mother to the daughter cell. In yeast, the vacuole/lysosome, which is inherited, has critical functions including maintaining hydrostatic pressure/pH, as well as recycling, storage and degradation of macromolecules. The daughter cell inherits the vacuole *via* the myosin V motor, Myo2, and the vacuole-specific adaptors, Vac17 and Vac8.

When vacuole inheritance is defective, the bud generates a new vacuole that is independent of the mother vacuole. Moreover, after cytokinesis, the new cell does not form a bud until its vacuole reaches a specific size. These results suggest that the vacuole is essential for cell viability. If true, then cells defective in both vacuole inheritance and in the *de novo* synthesis of a new vacuole, would not be viable. Indeed, *pep12 vac17* and *vps45 vac17* double mutants exhibit synthetic growth defects. That the Pep12, t-SNARE and Vps45, Sec1/Munc18-like molecule are necessary for transport to the pre-vacuolar compartment, suggests that when a mature vacuole is not inherited, Pep12/Vps45 are required for maturation of endosomes to a fully functional vacuole. Furthermore, these double mutants show a G1 cell-cycle arrest, indicating that the vacuole is required for cell-cycle progression. A clue to why the vacuole is required was revealed by the finding that the *tor1 vac17* double mutant shows synthetic growth defects. The Tor1 protein kinase is a member of the TORC1 complex, which signals from the vacuole. In further studies we found that when a vacuole is not inherited, the newly synthesized vacuole cannot recruit the major TORC1 target, Sch9. It is tempting to speculate that the newly synthesized vacuoles lack additional properties essential for normal vacuole function. Taken together, these results suggest that a functional vacuole is required for cell-cycle progression in part through the TORC1 pathway.

P394**Board Number: B536****STX13 controls the melanosome biogenesis through its regulated endosomal fusion events.**R.A. Jani¹, L. Purushothaman¹, E. Sviderskaya², D. Bennett², G. Raposo³, S. Gangi Setty¹;¹Indian Institute of Science, Bangalore, India, ²Biomedical Sciences Research Centre, St. George's, University of London, London, UK, ³Centre de Recherche, Institut Curie, Paris, France

Melanosome biogenesis is a highly regulated organelle maturation process wherein an immature compartment acquires its cargo proteins through the endocytic trafficking pathways and finally matures into a functional organelle. These processes were shown to be dependent on several protein complexes

like AP-1, AP-3, BLOC-1,2,3 (Biogenesis of Lysosome-related Organelles Complex) and in addition to KIF13A motor protein and RAB32/38 GTPases. However the cargo delivery to any membrane requires the interplay between Rabs, tethering factors and SNAREs. Mutations in these proteins result into defective melanosome maturation leading to Hermansky-Pudlak syndrome (HPS), characterized by hypopigmentation, pulmonary fibrosis, bleeding disorders and other phenotypes.

Endosomal SNARE, STX13 was known to interact with BLOC-1, however the functional role of this interaction in pigment formation is unknown. Previous studies have shown that in AP-3-deficient melanocytes this SNARE mislocalizes to melanosomes, suggesting a role for AP-3 in recycling the STX13 from melanosomes. Bioinformatic analysis of the N-terminal region of STX13 revealed two canonical motifs, ³YGP⁶L resembling the classical tyrosine-based motif (YXX ϕ) and KETNE⁸⁰L⁸¹L resembling a dileucine motif [DE]XXXL[LI] recognized by several adaptor proteins. Mutagenesis analysis of these motif had no effect on the steady state distribution of STX13 indicating that STX13 uses non-canonical residues for its sorting or recycling. Further deletion of the N-terminal region (1-129 aa) redistributes the STX13 to melanosomes. In addition we found that STX13 mediated cargo delivery to the melanosomes is affected in the VAMP7 knockdown cells. The knockdown of either VAMP7 or STX13 affect the cargo trafficking and SNARE localization to the melanosomes, Taken together our findings reveal the importance of SNARE dynamics in melanosome biogenesis.

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Board Number: B537

Melanoregulin may influence melanosome distribution through its effects on the organization of lipids in the melanosome membrane.

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In mammals, pigments are made by melanocytes within a specialized organelle, the melanosome. Mature, pigment-laden melanosomes are then transferred to keratinocytes to drive the visible pigmentation of the animal's hair and skin. The dilute suppressor (*dsu*) locus is an extragenic suppressor of the pigmentation defect exhibited by mice lacking myosin Va (i.e. dilute mice). We recently showed that (i) melanoregulin, the product of the *dsu* locus, functions as a negative regulator of a shedding mechanism that drives the intercellular transfer of melanosomes from the melanocyte to the keratinocyte (Wu et al, PNAS, 2012), and (ii) that melanoregulin is stably anchored in the melanosome membrane through palmitoylation (Wu et al BBRC, 2012). An alternate model for melanoregulin function posits that the protein serves as the receptor on the melanosome membrane for dynein by recruiting the Rab7 effector RILP, which in turn recruits the dynein/dynactin complex (Ohbayashi et al, JCS, 2012). Consistently, over expression of melanoregulin drives melanosomes towards the MTOC. While we also find that over expression of melanoregulin drives melanosomes (and lysosomes in generic cells) towards the MTOC, we think this is a nonphysiological phenomenon due to an effect that excess melanoregulin may have on the organization of lipids in the melanosome membrane by virtue of melanoregulin's multiple attached lipids (myristate and up to six palmitates). Moreover, we find that

the ability of melanoregulin to promote the minus end directed movement of melanosomes/lysosomes requires Rab7 as well as RILP. Finally, while artificial anchoring of RILP in the mitochondrial outer membrane drives the organelle to the MTOC, artificial anchoring of melanoregulin does not. We conclude that melanoregulin does not serve in place of Rab7 as the anchor for the RILP/dynein/dynactin complex on the surface of melanosomes. Ongoing studies are addressing how melanoregulin's attached lipids, when present in excess, influence the kinetics of the melanosome's microtubule minus end-directed transport.

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Board Number: B538

SNARE protein requirements for lytic granule exocytosis in immune cells.

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Cytotoxic T Lymphocytes (CTLs) and Natural Killer (NK) cells selectively kill virally infected or cancer cells by releasing the lytic granule content at the contact area with target cells called immunological synapse (IS). SNARE proteins are required for multiple membrane trafficking steps to mediate the maturation and final exocytosis of "fully-armed" lytic granules at the IS. Deregulation of SNARE-mediated fusion events, such as in Familial Hemophagocytic Lymphohistiocytosis-4 and -5, in which Syntaxin-11 and Munc18-2 are mutated, respectively; severely impair lytic granule release and cytotoxicity of CTL and NK-cells. Syntaxin-11 and Munc18-2 have also been shown to be essential for platelet granule secretion and other important physiological processes. However the underlying molecular mechanisms by which Syntaxin-11 and Munc18-2 control membrane trafficking and lytic granule exocytosis are still elusive. We have identified that endogenous SNAP23 and Vamp8 physically interact with Syntaxin-11 in primary CTLs. Biochemical experiments showed that these interacting proteins can form stable SNARE complexes and confirmed the specificity of these interactions. Stimulated Emission Depletion (STED) Superresolution microscopy studies positioned these SNARE proteins at the central stage to control granule exocytosis. Two pools of Syntaxin-11 were identified in CTLs, one localized to a vesicular compartment, and one localized at the plasma membrane. Suggesting that Syntaxin-11 is involved in an active recycling through the endosomal compartment. In contrast, Vamp8 was mapped to internal vesicles partially colocalizing with lytic granule markers such as Perforin and Granzyme. Cell-cell fusion experiments revealed that flipped-Stx11/SNAP23 t-SNARE complex specifically promoted membrane fusion with flipped-Vamp8 but not with flipped-Vamp3, 4 or 7. These results support the conclusion that the identified Stx11/SNAP23/Vamp8 SNARE complex can act as fusogenic SNARE complex and may play a crucial role during CTL-mediated cytotoxicity and FHL pathophysiology.

P397**Board Number: B539****A massive ectopic accumulation of lysosomes in axons that surround amyloid plaques is a defining feature of Alzheimer's disease.**S. Gowrishankar¹, P. Yuan², Y. Wu^{1,3,4}, S. Paradise¹, J. Grutzendler², P. De Camilli⁵, S.M. Ferguson¹;¹Department of Cell Biology, Yale University, New Haven, CT, ²Department of Neurology, Yale University, New Haven, CT, ³department of cell biology, 1Howard Hughes Medical Institute, New Haven, CT, ⁴CNNR program, Yale University, New Haven, CT, ⁵Department of Cell Biology, Howard Hughes Medical Institute, Program in Cellular Neuroscience, Neurodegeneration and Repair, Yale University School of Medicine, New Haven, CT

A key feature of Alzheimer's disease (AD) brain pathology is the presence of amyloid plaques. These are extracellular aggregates of the amyloid β peptide surrounded by swollen neuronal processes (dystrophic neurites). To address the molecular mechanisms underlying this aspect of Alzheimer's disease pathology, we pursued an extensive characterization of the amyloid plaques and their effects on surrounding brain tissue. We observed that axons passing in close proximity to extracellular amyloid deposits are filled by focal accumulations of lysosomes (as indicated by the presence of multiple peripheral, transmembrane and luminal proteins that molecularly define this organelle). Prominent among these lysosomal components is progranulin, a protein of the lysosomal lumen that is linked to neurodegenerative disease. The massive abundance of lysosomes in dystrophic axons adjacent to extracellular amyloid deposits stands in striking contrast to the predominant localization of lysosomes to the perikarya and dendrites of normal neurons. Thus, both the accumulation of lysosomes around A β deposits, as well as the abundance of lysosomes in axons represent a major, defining feature, of the neuropathology that accompanies amyloid plaque formation. The close relationship between amyloid plaques and axonal lysosome accumulation was observed from the earliest detectable stages of development of these deposits and precedes the recruitment of microglia to plaques. β -secretase (an essential enzyme in the production of A β) also colocalized with the lysosomal marker LAMP1 in axonal swellings, raising the possibility of a feed-back loop between extracellular A β deposits, axonal lysosomal traffic and the local production of A β . Further elucidation of the contributions of abnormal dynamics of lysosomes in Alzheimer's disease may yield important insights that point to new therapeutic targets for the disease.

P398**Board Number: B540****Identification of a Mutation in a Novel Gene Causing a Chédiak-Higashi Syndrome-Like Phenotype.**

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Chédiak-Higashi syndrome (CHS) is a rare autosomal recessive disorder characterized by partial albinism, recurrent infections, a mild bleeding tendency, and later onset neurological involvement including learning difficulties, cerebellar dysfunction, polyneuropathies, and L-DOPA responsive Parkinsonism. CHS was diagnosed based on identification of enlarged granules on peripheral blood smear and enlarged lysosomes and lysosome-related organelles in many cell types. Previously, CHS was known to be caused only by mutations in the lysosomal trafficking regulator gene (*LYST*), which encodes a cytosolic protein proposed to function in the regulation of lysosomal size and trafficking. Some patients with CHS do not have mutations in *LYST*, suggesting that mutations in other genes could also result in a similar disorder. Here we describe a set of twins of consanguineous background who presented with hepatosplenomegaly and were diagnosed with CHS based on typical inclusions in peripheral leukocytes and in precursor cells in the bone marrow. Their subsequent clinical course was dominated by severe global developmental delay and refractory epilepsy; at age 6 years they were non-verbal and unable to sit unsupported. Cellular analysis showed enlarged lysosomes that clustered in the perinuclear regions of both patients' fibroblasts. Whole exome sequencing of one of these patients revealed no mutations in *LYST*, but identified a homozygous 1bp deletion in *WDR91*, representing a novel gene causing a CHS-like phenotype. The same mutation was identified in an affected sibling by Sanger sequencing, and segregated with disease status within the family. *WDR91* encodes WD40 Repeat 91 protein that contains several WD40 domains similar to *LYST*. These domains fold into a beta-propeller structure known to form a platform that mediates protein-protein interactions. Consistent with the severity of the mutation, *WDR91* protein expression is completely absent in the two affected individuals and likely accounts for their CHS-like phenotype. Identification of other CHS patients with *WDR91* mutations will help us understand if *WDR91*-related CHS causes the severe neurological phenotype observed in these siblings.

P399**Board Number: B541****SPPL3 cleaves CLN5 to produce a mature soluble lysosomal protein.**E. Sauvageau¹, F. Jules², D. Scott³, K. Dumaresq-Doiron³, S. Lefrançois²;¹Nephrology, Hopital Maisonneuve-Rosemont, Montreal, QC, ²Hopital Maisonneuve-Rosemont, Montreal, QC, ³Universite de Montreal, Montreal, QC

Neuronal ceroid lipofuscinoses (NLCs) are a group of recessive disorders of childhood that lead to vision loss, dementia, ataxia and premature death. 14 different genes have been linked to NCLs (CLN1-CLN14), but the functions of the proteins encoded by the majority of these genes have not been fully elucidated. Mutations in the CLN5 gene are responsible for the Finnish variant late-infantile form of NCL (Finnish vLINCL). Functionally, CLN5 is implicated in the recruitment of the retromer complex to endosomes, which is required to sort the lysosomal sorting receptors from the endosomes to the trans-Golgi network. CLN5 is translated as a 407 amino acid type II transmembrane domain containing protein that is heavily glycosylated and subsequently cleaved into a mature soluble protein. The importance of CLN5 processing on its functions and the mechanisms that cleave CLN5 into a mature soluble protein are currently unknown. We sought to identify the enzyme(s) responsible for CLN5 cleavage by testing the effect of various protease inhibitors on CLN5 processing. We found that only (Z-LL)2-ketone prevented the cleavage of CLN5 from the pro form to the mature form as the other inhibitors had no effect. (Z-LL)2-ketone inhibits the SPP family of intramembrane proteases that are known to cleave a variety of type II transmembrane proteins. To determine which member(s) of the SPP family is responsible for CLN5 processing, we overexpressed WT or catalytically dead mutants of the SPP family (SPP, SPPL2A, SPPL2B and SPPL3) and looked at their consequences on CLN5 cleavage. We found that only the catalytically dead SPPL3 was able to block CLN5 conversion from the pro to the mature form. To confirm these results, we individually knocked down the expression of each SPPs and looked at the effect on CLN5 processing. Again, only shRNA targeting SPPL3 were able to prevent the cleavage of CLN5 from the pro to the mature form. We also found that the N-terminal fragment resulting from CLN5 processing is subsequently cleaved by another member of the SPP family and degraded by the proteasome. In order to identify the cleavage site, we overexpressed CLN5-HA in HEK 293 cells, purified CLN5 using an anti-HA antibody and sent the purified protein for mass spectrometry analysis. Two potential cleavage sites were identified but so far mutations around these cleavage sites have not block CLN5 processing. Herein, we show that SPPL3, a member of the SPP intramembrane protease family, cleaves CLN5 into a mature soluble protein and a N-terminal fragment, which is subsequently cleaved by SPPs. This work further characterizes the biology of CLN5 in the hopes of identifying a therapeutic strategy.

P400**Board Number: B542****Functional characterization of Atg20 and Atg24 domains in autophagy regulation.**Y. CHEN¹, L. YU¹, W. HUANG¹;¹DEPARTMENT OF LIFE SCIENCE, NATIONAL TAIWAN UNIVERSITY, TAIPEI, Taiwan

Autophagy is a stress response mechanism that delivers cytosolic materials to the vacuole/lysosome for degradation and recycling. Because autophagy is highly activated by nutrient-starvation stresses, it had been considered simply as a non-specific catabolic pathway for a long time. Recent studies, however, have uncovered intimate relationships between selective autophagy activities and human diseases, such as pathogen infections and tissue-degenerative diseases. Based on results of animal model studies, autophagy is also proposed as a mechanism to prevent aging by eliminating dysfunctional mitochondria. All these newly reported autophagy functions rely on efficient signaling mechanisms to recognize and segregate specific cargoes for degradation, which makes the selective autophagy regulatory machinery a potential target for therapeutic manipulations. Working on a suitable model system should accelerate our studies on the molecular mechanisms of selective autophagic cargo-induced signaling processes. Several selective autophagic cargoes have been uncovered in the budding yeast *Saccharomyces cerevisiae*. Among them, the precursors of aminopeptidase I enzyme (prApe1) are delivered directly from cytosol to the vacuole through the actions of autophagy proteins under nutrient-rich growth condition. This makes prApe1 the only model for the study of the induction signaling of selective autophagy pathways in nutrient-rich environment. Because the other types of selective autophagy pathways are stimulated by nutrient-starvation conditions, which also induce non-selective autophagy activities, this makes the attribution of observed molecular events for selective or non-selective autophagy regulation difficult. We report our results on characterizing the functional domains of two BAR-domain proteins, Atg20 and Atg24, in prApe1-stimulated selective autophagy regulation.

P401**Board Number: B543****Elevated levels of FTD risk factor TMEM106B alter lysosomal/autophagosomal pathways.**J.I. Busch¹, T.L. Unger¹, R.T. Skrinak¹, N. Jain¹, W. Mayes¹, A.S. Chen-Plotkin¹;¹Neurology, University of Pennsylvania, Philadelphia, PA

Frontotemporal lobar degeneration with TDP-43 inclusions (FTLD-TDP) is an important cause of dementia in individuals under 65 years old. Common variants in the Transmembrane Protein 106B gene, or *TMEM106B*, were discovered by genome-wide association to confer genetic risk for FTLD-TDP ($p=1 \times 10^{-11}$, OR=1.6). Evidence suggests that *TMEM106B* risk variants increase one's probability for developing FTLD-TDP by increasing *TMEM106B* expression. Prior to its association with FTLD-TDP, *TMEM106B* was largely uncharacterized. *TMEM106B* has subsequently been demonstrated to be widely

expressed in the brain, with a perikaryal distribution in neurons. Moreover, TMEM106B is a Type II transmembrane protein with steady-state localization to lysosomes.

To better understand TMEM106B's role in FTLT-DTP pathophysiology, we investigated the effects of increased TMEM106B expression in multiple cell types, including primary neurons. Increased TMEM106B expression resulted in a striking cellular phenotype - extremely enlarged organelles (>3 μ m) that were positive for the lysosomal marker LAMP1. Ultrastructural analysis suggested that these organelles were late autophagic vacuoles (amphisomes/autolysosomes). Accompanying these morphological changes were alterations in autophagy and lysosomal pathways. Specifically, increasing TMEM106B expression resulted in a significant increase in the number of autophagosomes and autolysosomes. This was accompanied by the loss of highly acidified organelles, a decrease in the total number of LAMP1+ organelles, and impaired lysosomal degradation. Taken together, these results suggest loss of a functional lysosomal pool.

The cell biological effects of increased TMEM106B expression were dependent on lysosomal targeting of TMEM106B, since a point mutation in a lysosomal sorting motif abrogated these effects. These effects were also dependent on the presence of C9orf72 protein, since C9orf72 knockdown rescued these effects as well. Of note, hexanucleotide repeat expansions in *C9orf72* are the most common Mendelian cause of both FTLT-DTP and ALS. In addition, we have previously shown that *TMEM106B* acts as a genetic modifier affecting age at death in *C9orf72* expansion-associated FTLT-DTP. **Our results thus suggest that TMEM106B exerts its effects on disease risk through alteration of lysosomal and autophagy pathways. In addition, TMEM106B and C9orf72 may interact in the pathophysiology of FTLT-DTP.**

P402

Board Number: B544

Subcellular targeting of natural and engineered anti-autophagy RavZ proteins.

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Autophagy involves the capture and delivery of cytosolic cargo to the lysosome for degradation, and is a key mediator of the cellular host response. Pathogens in turn, have developed mechanisms to evade the cellular host response, and we have recently described the Legionella effector RavZ as a potent inhibitor of autophagy. Upon infection, RavZ is secreted into the cytoplasm where it cleaves the lipidated LC3 protein but not the soluble form. Consistent with this biochemical function, here we show that RavZ cleaves LC3 upon starvation in HeLa and the mouse RAW 264.7 macrophage cell line. To investigate the cellular consequence of local LC3-PE depletion, we designed membrane-tethered RavZ constructs, potentially limiting RavZ activity to selective sites within the cell. First, we fuse RavZ to the endoplasmic reticulum (ER)-binding domain of cytochrome b5 (cb5(ER)). RavZ-ER co-localizes with the ER marker PDI. RavZ-ER cells show drastically reduced number of LC3 puncta, and reduced LC3-II

formation, consistent with starvation-dependent autophagosome formation occurring in close proximity to ER membranes. Next, we have generated tools for targeting RavZ to mitochondria using the mitochondrial form of cb5. I will present data exploring the effects of these two membrane-limited constructs on starvation-induced autophagy and on the more peripheral process of LC3-associated phagocytosis (LAP). We also previously showed that the catalytically-inactive point mutant of RavZ, RavZ(C258A), co-localizes with LC3 puncta. Anchored RavZ(C258A) may therefore recruit LC3 to target membranes, and I aim to apply FPALM microscopy to study the distribution of LC3 over large cargo such as mitochondria, ER and in cells undergoing LAP. Preliminary experiments on starved cells show LC3-decorated cup- and sphere-shaped structures less than 300nm in diameter. In summary, I have generated unique tools that allow us to independently manipulate autophagy subtypes. Using these tools, we are able to investigate how RavZ targets membranes, and its role in LC3-associated phagocytosis. Our preliminary studies using FPALM furthermore pave the way for understanding the organization of LC3 and upstream factors at the autophagic membrane.

P403

Board Number: B545

Atg4 mediated release of LC3-PE is acutely sensitive to the composition and organization of the surrounding membrane.

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Autophagy is a bulk degradation process in which material to be degraded is encapsulated in a double-membraned organelle called the autophagosome and delivered to the lysosome. The growth and maturation of the autophagosome depends upon the transient attachment of LC3 family proteins to the phospholipid headgroups of the growing autophagosome. Soon after translation, proform LC3 is cleaved by the protease Atg4 to reveal a glycine residue that is conjugated to the lipid phosphatidylethanolamine (PE) in a ubiquitin-like process. Atg4 also functions later in autophagy, cleaving LC3 back off of the lipid headgroups to allow the autophagosome to fuse with the lysosome. Each Atg4-dependent cleavage is essential; if lipidation is prevented the autophagosome does not fully mature and close, while if LC3 is never released from the autophagosome membrane, the autophagosome is unable to fuse with the lysosome to complete autophagy. Therefore, the lifetime of lipid conjugated LC3 must be regulated, but how these lipidation and delipidation rates are controlled is unknown. Here we apply in vitro reductionist methods to determine the intrinsic activities of Atg4 against soluble precursor and mature lipidated forms of LC3. We show that Atg4-mediated release of lipidated LC3 is an order of magnitude slower than processing of soluble precursor LC3. The differences in processing kinetics do not derive from differences in recognizing the protein-lipid adduct per se, but instead is due to the anchoring of the substrate LC3-PE in a bilayer membrane; simply solubilizing the membrane with detergent allows LC3-PE to be processed as quickly as fully proteinaceous soluble LC3. Further, the release of LC3-PE is sensitive to changes in membrane composition, suggesting that the lifetime of lipidated LC3 may be controlled by local alterations in membrane structure. The recognition

of soluble and lipidated substrates differs among Atg4 homologs; Atg4A is much more efficient at delipidation than Atg4B. As Atg4A is only able to process the GABARAP subfamily of LC3 proteins, the available pool of GABARAP lipidated protein may also be under distinct temporal controls from the pool of LC3 lipidated protein. Collectively, our results suggest that Atg4 is naturally tuned to work slowly on early autophagosome intermediate membranes allowing LC3-PE (or GABARAP-PE) to accumulate to high levels during autophagosome growth.

P404

Board Number: B546

Endosomal pH in c-Met Trafficking.

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Up-regulation of the receptor tyrosine kinases (RTKs) signalling cascades constitutes the basis of tumorigenesis. Signal termination that normally occurs through endosomal pH dependent ligand-receptor dissociation and receptor degradation is disrupted in various cancers. Therefore, persistent receptor-mediated signal occurs through increased recycling and elevated surface expression of these receptors. In this study, we show that neuron-enriched Na⁺/H⁺ exchanger NHE5 plays an important role in trafficking of c-Met RTK in C6 glioma cells. NHE5 is predominantly expressed in recycling endosomes of C6 cells and localizes partially to transferrin(Tfn) and Rab11 positive endosomes. By fluorescence ratiometric analysis, we show that NHE5-depletion with short-hairpin RNA significantly increases pH of Tfn positive recycling endosomes, suggesting a prominent role of NHE5 in endosomes acidification. Using biochemical approach, we show that cell surface abundance of hepatocyte growth factor (HGF) receptor c-Met is reduced by 80% in NHE5-deficient cells as a result of impaired recycling circuitry. Consequently, reduced expression of c-Met by NHE5 knockdown (KD) causes severe migratory defect and loss of cell polarity. We further demonstrate that disrupted phenotypes in NHE5 KD cells are restored by NHE5 complementation. Stable expression of shRNA-resistant human NHE5 into NHE5 KD cells acidifies endosomal pH, increases surface c-Met abundance and Rac-1 activity, and enhances cell migration. In summary, we showed that acidification of recycling endosomes by NHE5 promotes recycling of c-Met, which contributes to persistent downstream signalling cascade and enhancement of cell motility. Our study highlights critical role of endosomes pH in regulating receptor-mediated signalling through vesicular trafficking.

P405**Board Number: B547****Pathophysiology of Morbus Niemann-Pick Type C1: Membrane lipid abnormalities in cultured fibroblasts and lymphoblasts.**S. Rizk¹, K. Maalouf¹, M.H. Hodroj¹, A. Das², H.Y. Naim³;¹Lebanese American Univ, Beirut, Lebanon, ²Department of Pediatrics, Hannover Medical School, Hannover, Germany, ³Department of Physiological Chemistry, University of Veterinary Medicine Hannover, Hannover, Germany

Morbus Niemann–Pick type C1 (NPC1) is a rare, life-threatening, autosomal-recessive, neurovisceral lysosomal storage disease (LSD). It is characterized by a cellular cholesterol trafficking defect which leads to the entrapment of cholesterol and glycosphingolipids inside lysosomes. The pathophysiology of clinical symptoms in NPC1 patients is not clear. As cholesterol is a pivotal membrane component the function of biological membranes may be disturbed, specifically in the ordered structure of a particular type of membranes, detergent-resistant membranes (DRMs) and /or lipid rafts, which are enriched in glycosphingolipids and cholesterol. In cultured skin fibroblasts and lymphoblasts harboring NPC1 mutations and cells from healthy individuals (WT cells), lipid rafts were analyzed by isolating TritonX-100 DRMs and separating them on a sucrose density gradient. The distribution of the lipid rafts-associated protein flotillin2 was shown to be altered in both cell lines relative to WT cells. In contrast, the distribution of RhoA, a protein that is not associated with lipid rafts, did not change between NPC1 B-lymphocytes and fibroblasts relative to WT cells. Protein array studies were done on both NPC1 B-lymphocytes and fibroblasts in comparison with normal WT cells. In both cell lines cytochrome C as well as the heat shock proteins, HSP60 and HSP70, appeared to be less expressed in NPC1 compared to WT cells. Together, these results provide a promising basis for understanding the pathophysiology in NPC1 and propose potential biomarkers.

P406**Board Number: B548****GALECTIN-8 IS SECRETED IN APICAL EXOSOMES FROM MDCK CELLS.**C. Oyanadel¹, M. Lopez-Verrilli¹, A. Soza¹, A. Gonzalez¹;¹Inmunología Clínica y Reumatología. Fac. de Medicina. Centro de Envejecimiento y Regeneración, Fac. Ciencias Biológicas, P. Universidad Católica de Chile, Santiago, Chile

Galectin-8 belongs to a family of carbohydrate-binding proteins secreted by a nonconventional route and implicated in a variety of cellular processes, including adhesion, migration and apoptosis. Galectins are involved in apical secretion from polarized epithelial cells. Cancerous cells derived from epithelial cells have altered expression of Gal-8. Here, we generated MDCK cells permanently transfected to express Gal-8 and studied its secretion mechanism. Gal-8 was captured and precipitated with lactose-Agarose beads from the media in both steady state and pulse-chase experiments. Exosomes were isolated by successive ultracentrifugation from conditioned media of MDCK cells grown on tissue culture

dishes and Transwell filters. After 30 min of pulse labeling we found newly synthesized Gal-8 only in the apical medium. The exosome fraction characterized by the presence of CD63, Hsp70, Hsp90 and flotillin markers contained Gal-8. We detected Gal-8 resistant to trypsin digestion indicating its presence inside the exosomes. However, we detected a proportion of Gal-8 in the surface of exosomes by competition with 50 mM lactose and by immunoelectron microscopy. SDS-PAGE silver stained pattern showed differences in apical and basolateral exosomes. In addition, apoptosis induction of activated T cells used as a described test showed evidence that apically secreted exosomes containing Gal-8 are biologically active. The selective PIKfyve inhibitor described to induce accumulation of late endosomal compartments, presumably multivesicular bodies, decreased the secretion of Gal-8. All these results indicate that MDCK cells secrete apical exosomes containing Gal-8 originated at late endosomal compartments (Financial support from: FONDECY# 1141127 and CONICYT grant PFB12/2007).

P407

Board Number: B549

Phagosome formation in Choanoflagellates (*Salpingoeca rosetta*).

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The link between unicellular life and multicellular animals has extensive implications in molecular and cellular biology. The emerging field of evolutionary cellular biology sheds light on this link by examining the physiological and anatomical similarities between the unicellular protists, choanoflagellates, and choanocytes, cells found in sponges in the phylum Porifera. The physical similarities between these cells are striking, but in addition, these cells are astonishingly similar in their function when choanoflagellates are in their colony form. Both choanocytes and choanoflagellates use a flagellum to create a water current that facilitates phagocytosis of prey. In colonies, the flagellum of each choanoflagellate radiates outward from the circle formed by the cells, creating a current similar to that made by the choanocytes of a sponge. We are interested in comparing phagocytic vacuole formation in single cell and colony forms. We suspect that phagosome formation in colonies will be more rapid and efficient than in single celled choanoflagellates. Single cells and colonies of *Salpingoeca rosetta* were incubated with BioParticles, which are heat killed, fluorescently labeled *Staphylococcus aureus*, and phagosome formation was quantified as phagosomes per cell in single cells and phagosomes per colony in colonies. Preliminary results showed that colonies formed 1.1 ± 0.15 phagosomes/cell (mean \pm SEM; N=23 colonies), compared to 0.51 ± 0.16 phagosomes/cell in single cells (N=37). The average number of cells per colony was 6.17 ± 0.40 (N=23). These results suggest that choanoflagellate colonies are more efficient at forming phagosomes than single cells.

P408**Board Number: B550****Induced pluripotent stem cell model of mucopolysaccharidosis type II.**R. Dobrovolny¹, J. Rybova¹, H. Poupetova¹, L. Kuchar¹, B. Asfaw¹, J. Ledvinová¹;¹Institute of Inherited Metabolic Disorders, First Faculty of Medicine, Charles University in Prague, Prague, Czech Republic

Mucopolysaccharidosis type II (MPSII, hunter syndrome) belongs to a family of inborn lysosomal storage disorders. The defect is caused by X-linked (IDS, Xq28) deficient activity of lysosomal iduronate-2-sulfatase (I2S) leading to an accumulation of undegraded glycosaminoglycans (GAG) in tissues throughout the body. The clinical symptoms appear in early childhood typically as ear and respiratory tracts infections, abdominal hernias and later progressively develops in severity to facial dysmorphies (a prominent forehead, a nose with a flattened bridge, and an enlarged tongue), organ involvement (enlarged liver, cardiomyopathy, airways thickening, joint stiffness) and mental and behavioral decline. The life expectancy of classical male patients is 10-20 years. The main accumulated GAGs are dermatan sulfate and heparin sulfate. The presence of these GAG in urine, deficient I2S activity in leukocytes, clinical features and finally demonstration of pathological mutation in IDS gene are the basis of the diagnosis. Enzyme replacement therapy (ERT) with idursulfase (Elaprase®), a recombinant form of human iduronate 2-sulfatase, has recently been approved in the United States and the European Union. However, only attenuated, non-neurological, form of the disease is considered for ERT as the enzyme does not cross the blood brain barrier. Mouse model of MPSII, replicating human pathology, was found useful for development of ERT and pathogenesis studies on organism level. However, it has been repeatedly shown that mouse models often reflect human disorders incompletely. Use of human tissue cultures can help to overcome some drawbacks of mouse material; however it is usually limited only to accessible cell types such as skin fibroblast. Here we report generation of induced pluripotent stem cell lines from MPSII patients. These cell lines were generated by non-integrating Sendai virus protocol (CytoTune®, LifeTechnologies). The cells expressed pluripotency markers and can be differentiated to all three germ layer. The accumulation of specific GAG was tested by immunocytofluorescence and electrophoresis. The established cellular model can be used to further study the pathobiochemistry of MPSII and novel therapeutic approaches for the disease.

P409**Board Number: B551****Fabry cardiomyocytes generated from induced pluripotent stem cells as a human model for morphology and pathobiochemistry studies.**J. Rybová, R. Dobrovolný¹, B. Asfaw¹, J. Sládková², M. Hyliš³, J. Ledvinová¹;¹Institute of Inherited Metabolic Disorders, First Faculty of Medicine, Charles University in Prague, Prague, Czech Republic, ²Department of Pediatrics and Adolescent Medicine, First Faculty of Medicine, Charles University in Prague, Prague, Czech Republic, ³Laboratory of Electron Microscopy, Faculty of Science, First Faculty of Medicine, Charles University in Prague, Prague, Czech Republic

Lysosomal storage disorders is a group of more than 60 genetic disease caused by functional defects in lysosomal enzymatic or non-enzymatic proteins. Fabry disease, an example of one enzymopathie, is caused by deficiency of lysosomal alpha-galactosidase A (EC 3.2.1.22). This defect leads to progressive accumulation of glycosphingolipids with terminal alpha-galactosyl moiety, dominantly globotriaosylceramide (Gb3Cer) and smaller amounts of galabiosylceramide (Ga2Cer) and blood group B antigens in lysosomes of most non-neuronal tissues (mainly heart, kidney and liver) and body fluids [Elleder in Fabry disease, Springer 2010]. Induced pluripotent stem (iPS) cells are generated from somatic cells by their reprogramming with four transcription factors Oct3/4, Sox2, c-Myc and Klf4 [Takahashi et al, 2006]. These cells provide an excellent opportunity to produce disease-relevant cell types for pathophysiology studies and development of new therapeutic approaches. We prepared transgene-free human iPS cells from urine cells isolated from Fabry patients by transduction with Sendai virus expressing reprogramming factors. Derived iPS cell lines were positive for pluripotent markers (Lin-28, Oct3/4 etc.) and alkaline phosphatase activity. Fabry disease phenotype was confirmed by determining deficient alpha-galactosidase activity. Cardiomyocytes (CM) are one of the major cell types affected in Fabry disease and therefore we decided to differentiate generated iPS cells into cardiac cells. We performed differentiation based on the work of Lian et al [Lian et al, 2012]. Generated Fabry-CM and control-CM were positive for cardiac markers (Troponin I, Nkx2.5). The efficiency of differentiation, tested by flow cytometry, was significantly smaller for Fabry-CM compared to control probably due to Fabry phenotype. Ultrastructure analysis of Fabry-CM revealed abnormally sized and shaped mitochondria, almost regularly associated with aberrant and sparse cristae, dilated endoplasmatic reticulum and membranous cytoplasmic body structures in comparison with controls. We suggest that observed damages of intracellular architectures of Fabry-CM are tightly associated with their functional pathology followed by clinical phenotype of the heart of Fabry patients, predisposed to storage. Other studies are needed to elucidate the cascade of molecular events connected with etiopathogenesis FD. Recently, it was described effective clearance of Gb3Cer by the recombinant enzyme in Fabry disease iPS cell [Iltier et al, 2014]. Trafficking of supplied enzyme within the compartments highly loaded with storage compounds will be the subject of our further studies.

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P410**Board Number: B552****Mitotic Membrane Remodelling by the ESCRT machinery.**Y. Olmos^{1,2}, L. Hodgson³, J. Mantell³, P. Verkade³, J. Carlton²;¹Cancer Studies, King's College London, London, United States, ²Cancer Studies, King's College London, London, England, ³Univ Bristol-Sch Biochem, Bristol, England

The Endosomal Sorting Complex Required for Transport (ESCRT) Machinery regulates diverse membrane remodelling events that allow completion of cytokinesis, intraluminal vesicle biogenesis at the multi vesicular body and the release of enveloped retroviruses such as HIV-1. We employ here high resolution light and correlative electron microscopy to examine the localisation of ESCRT-III components through different phases of mitosis. We discover a new localisation and function for ESCRT-III in mitotic membrane remodelling during telophase and explore this function using collective 3D electron tomography.

P411**Board Number: B553****Activator of G-protein Signaling 3 (AGS3)-induced lysosomal biogenesis mediates resistance against (antibiotic-resistant) intracellular pathogens.**A. Vural¹, S. Al-Khodor², G.Y. Cheung³, C.S. Shi¹, L. Srinivasan⁴, V. Briken⁵, M. Otto³, I.D. Fraser², J.H. Kehrl¹;¹Laboratory of Immunoregulation, National Institutes of Allergy and Infectious Diseases, BETHESDA, MD,²Laboratory of Systems Biology, National Institute of Allergy and Infectious Diseases, Bethesda, MD,³Laboratory of Human Bacterial Pathogenesis, National Institute of Allergy and Infectious Diseases,Bethesda, MD, ⁴Univ of Maryland, College Park, MD, ⁵Univ Maryland, College Park, MD

Enhancing the host immune response at host-pathogen interface represents an advantageous alternative strategy to combat drug-resistant bacterial infections. One such intervention would be to enhance the activity of lysosomes, which are the ultimate degradation organelle for internalized bacteria. Here we report that increasing the levels of Activator of G-protein Signaling 3 (AGS3) augments lysosome activity, thereby limiting intracellular bacterial infections in macrophages. AGS3 is a guanine nucleotide dissociation inhibitor (GDI) that binds GDP bound G α subunits of the Gi/Go/transducin family of G proteins via its four G-protein regulatory (GPR/GoLoco) motifs. This stabilizes the G α subunit in its GDP-bound conformation. AGS3 has been implicated in a broad array of physiologic functions including neuronal differentiation, addiction and craving behavior, autophagy, membrane protein trafficking, metabolism, cardiovascular regulation, polycystic kidney disease, and leukocyte migration. We found that increasing the level of AGS3 in the human macrophage cell line, THP-1, led to an increased nuclear translocation of the transcription factor EB (TFEB), a known regulator of lysosomal biogenesis. This occurred as a consequence of enhanced mTOR activity and led to a significant increase in lysosomal mass. To test the impact of AGS3-induced lysosome enrichment on the response of macrophages to

bacterial infection, THP-1 cells with low or elevated levels of AGS3 (AGS3lo and AGS3hi) were infected with the intracellular pathogen, Burkholderia cenocepacia, an antibiotic-resistant gram negative bacterium that causes significant morbidity in cystic fibrosis and chronic granulomatous disease patients. THP-1 AGS3hi cells suppressed the intracellular replication of Burkholderia cenocepacia BC J2315 by enhancing lysosomal degradation and reducing bacterial escape from the endo-lysosomal pathway. Moreover, the infection of AGS3 deficient primary macrophages, which possessed a reduced lysosomal mass, resulted in a higher intracellular bacterial replication rate than was noted with wild type macrophages. Furthermore, the THP-1 AGS3hi macrophages better controlled two other bacterial infections. Mycobacterium tuberculosis replicated at a slower rate in THP-1 AGS3hi macrophages and Methicillin-resistant Staphylococcus aureus strain 252 induced a lower level of cellular apoptosis resulting in enhanced cell survival. Thus, by elevating their levels of AGS3 macrophages can upregulate lysosomal biogenesis decreasing their susceptibility to intracellular bacterial infection. Identifying the signals that control AGS3 expression may provide a mechanism to limit intracellular bacterial infections in macrophages and perhaps other cell types.

P412

Board Number: B554

Autophagy is essential for the survival and regenerative potential of hematopoietic stem cells.

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While autophagy helps adult hematopoietic stem cells (HSC) to withstand metabolic starvation, its function in maintaining blood production remains unknown. Conditional inactivation of autophagy in the blood system results in myeloid expansion with mild anemia and lymphopenia, and maintenance of HSC numbers. However, autophagy-deficient HSCs show increased proliferation with enhanced myeloid differentiation, and rapidly become exhausted following transplantation with loss of self-renewal and regenerative potential. Electron microscopy analyses reveal increased numbers of usually deformed mitochondria, expanded endoplasmic reticulum and golgi compartments with excess of small vesicles, and misshaped nucleoli. Microarray gene expression analyses confirm significant changes in metabolic, membrane, nucleus, and mitochondria pathways. We are currently performing metabolic analyses to determine whether accumulation of mitochondria could drive increased oxidative phosphorylation and production of reactive oxygen species (ROS), thereby inducing HSC proliferation and functional exhaustion. Our results demonstrate that autophagy is essential for HSC survival and normal blood regeneration.

ER and Golgi Transport

P413

Board Number: B555

Role of Sop4, an ER membrane complex (EMC) member in protein quality control.

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Protein biogenesis in the secretory pathway requires precise folding and targeting of nascent polypeptide-bound ribosome complex at the ER. Quality control is essential such that cells do not accumulate aggregates of misfolded and mislocalized proteins that are deleterious to the cell. Here, we studied the biogenesis of Yor1p, an ortholog of CFTR (Cystic Fibrosis Transmembrane Regulator) in yeast *S. cerevisiae*. Yor1p is an ABC transporter that confers oligomycin resistance to the cell and like CFTR, a single mutation (Yor1- Δ F670) causes misfolding and retention in the ER. A high throughput screen of Yor1- Δ F with the yeast gene deletion collection identified regulators of membrane protein biogenesis (Louie, RJ et al., *Genome Med* 2012). The ER membrane complex (EMC 1-6) family along with Sop4 was identified as one of the deletion enhancers (slower growth on oligomycin) which indicated that these factors might promote Yor1p biogenesis. Interestingly, deletion of *sop4* did not affect stability of Yor1- Δ F highlighting its' role in the early stages of protein translation. Biochemical studies using short pulses of S35 revealed that Yor1p levels were indeed reduced in a *sop4* Δ strain compared to wild-type (wt). Similar results were seen in a double delete *emc6* Δ *emc2* Δ strain but not with individual deletions indicating that the EMC members might be functionally redundant. Further, deletion of ribosomal proteins such as *rpl12a* but not the cytosolic E3 ligase (*ltn1*), or the ERAD member (*cue1*) rescued growth on oligomycin and Yor1p levels were restored in the *sop4* Δ strain. Interestingly, in a *sop4* Δ strain, reduction in Yor1p levels was concomitant with elevated levels of the GPI-anchored protein, Gas1. Under these conditions, synthesis of the tail anchored protein, Sec22 wasn't affected. We propose two models to explain our findings: 1) Sop4 is an ER membrane chaperone which co-translationally regulates the biogenesis of membrane anchored proteins and likely communicates with the ribosome through associated proteins like *bfr1*, *scp160* and *rpl12a*. 2) Sop4 and the EMC complex mediate targeting of the ribosome-mRNA complex to the ER ensuring appropriate localization and translocation through the ER.

P414**Board Number: B556****ER export of yeast plasma membrane proteins requires dual interaction with the COPII coat.**S. Pagant¹, E.A. Miller¹;¹Biological Sciences, Columbia University, New York, NY

Many polytopic membrane proteins that reside at the yeast plasma membrane require the cargo receptor, Erv14, for efficient ER export. We recently found that the yeast ABC transporter, Yor1, similarly requires Erv14 for traffic despite having its own diacidic ER export signal. We sought to ask why a membrane protein that directly interacts with the Sec24 subunit of the COPII coat to facilitate ER export also requires a dedicated cargo receptor. Here we present a detailed structure-function analysis of Erv14 in the context of both Yor1 and a comprehensive array of additional polytopic membrane clients. We show that Erv14 indeed functions as a receptor, with a cargo-binding site contained within its second transmembrane domain that recognizes a subset of clients. The COPII binding signal of Erv14 is essential for traffic of all of its clients, further supporting a role as a classical cargo receptor. However, Sec24 itself seems to be the primary driver of ER export since overexpression of Sec24 could rescue Erv14 defects, at least with respect to Yor1 traffic. We propose that for a large class of polytopic membrane proteins, Sec24 binds to a dual signal that forms from two independent COPII binding signals: one on the cargo protein and one on Erv14, which in turn binds to cargo via a TMD-TMD interaction. This added affinity for the coat may improve packaging efficiency of large, bulky proteins that could be difficult to capture into a spatially constrained vesicle.

P415**Board Number: B557****Analysis of a yeast strain with deletion of multiple genes encoding yeast COPII vesicle proteins.**Y. Noda¹, S. Fujii¹, K. Yoda¹;¹Department of Biotechnology, The University of Tokyo, Tokyo, Japan

How a variety of newly synthesized membrane and secretory proteins are correctly and efficiently sorted into the COPII vesicles remains incompletely understood. To gain new insights into selective transport mechanisms at the ER and the Golgi, we created a yeast strain whose multiple genes encoding abundant COPII vesicle proteins are deleted simultaneously. The eight genes, *SVP26*, *ERV41*, *ERV46*, *ERV14*, *ERV29*, *EMP46*, *EMP47*, *GOT1*, were deleted by pop-in-pop-out replacement of the target genes using the *URA3* marker. The deletion strain of 8 genes (the $\Delta 8$ strain) showed decreased growth rate at 30°C, and was not able to form colonies at 16°C and 37°C. Introduction of the *ERV14* or the *GOT1* gene rescued the temperature-sensitive growth of the $\Delta 8$ strain, suggesting that the double disruption of the *ERV14* and *GOT1* genes is responsible for the growth retardation at high temperature. Although

the function of Erv14 as a cargo receptor for ER exit of a number of membrane proteins was already established (1)(2), the function of Got1, which is a conserved tetra membrane-spanning ER-Golgi protein, was totally unknown (3). Based on the result of our genetic analysis, we hypothesized that the Got1 may function to facilitate the ER-Golgi traffic of the membrane proteins either as a cargo receptor similarly to the other abundant membrane proteins in the COPII vesicles or by previously unappreciated mechanisms. To identify proteins that may interact with Got1 specifically either at the ER or Golgi, alanine substitutions were introduced into the three inter-transmembrane regions predicted to face the cytosol. We found that the alanine substitutions in the second inter-transmembrane region caused accumulation of the mutated Got1 protein in the ER, suggesting that this region may contain the sequence necessary for the efficient incorporation of Got1 into the COPII vesicles. The triple HA-tagged version of either wild-type or the mutated Got1 with alanine substitutions were immunoprecipitated and two co-immunoprecipitated proteins were found, one of which was specific for the wild-type Got1 protein, and the other one was specific for the mutated protein accumulated in the ER. These two proteins were identified using TOF-MS and we are currently investigating whether or not they have physiological roles in the biological processes where the Got1 protein functions. (1) Powers and Barlowe, Mol. Biol Cell. 2002 (13), p880, (2) Herzig, et al., PLoS Biol. 2012 (10), (3) Lorente-Rodríguez, et al., J. Cell. Sci, 2009, (122), p1540

P416

Board Number: B558

Live cell microscopy of early ER export events underpins a new model for COPII function at the ER-ER exit site boundary.

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ER exit sites (ERESs) are tubular-vesicular high curvature membrane domains scattered over the ER. These domains orchestrate the anterograde trafficking of membrane proteins from the ER to the Golgi complex by facilitating the selection and concentration of transport competent cargo proteins. These processes and the subsequent budding of vesicles are mediated by the cytosolic ERES membrane-associated COPII heterocomplex. Isoforms of the COPII subunit Sec24p recognize a broad variety of targeting signals at the cytosolic tail of membrane cargo proteins. The mechanistic details of how the COPII complex mediates the selection and concentration of correctly folded transport-competent cargo are obscure. Treatment of intact cells with BFA and Nocodazole uncouples early cargo recognition, sorting and concentration from later vesicle formation steps. Under these conditions ERESs lose their tubular-vesicular curved structure and become dilated spherical membranes. Nevertheless, using high temporal resolution microscopy and quantitative analysis we demonstrated that these dilated ERESs retain their ability to select and concentrate membrane cargo proteins in a COPII-targeting signal interaction dependent manner. In this experimental system we demonstrate that COPII, bound to a stem-like membrane structure connecting ERES to the ER, generates a flow of cargo proteins and lipids

into the sphere shaped ERES membrane. High-resolution multifocal SIM analysis (improved X-Y and Z resolution) confirmed the cylindrical shape of COPII coated membranes. Furthermore, high-speed confocal microscopy analysis indicates that the COPII coat exerts a diffusion barrier for exchange between the ERES and the ER lumens. Site directed mutagenesis of Sec24p was used to modify and increase the interaction between the acidic motif of VSVG and the Sec24B isoform. We anticipated that rather than accumulation of COPII coated ERES membranes, ER export of VSVG will be blocked at an earlier stage. Indeed, in cells expressing the mutant COPII subunit Sec24B-V923R cargo accumulation within ERES was significantly reduced resulting in cargo being mostly retained in the ER. These data are consistent with the premise that the Sec24B-V923R remains coupled to its VSVG cargo thereby preventing its flow into ERESs membranes. Our data supports the hypothesis whereby the COPII coated short tubular structure on the ER-ERES boundary serves as a quality control and cargo sorting and concentration complex. The cargo protein and membrane flow, generated by this COPII apparatus is facilitated by three driving forces: (1.) dynamic weak interactions between the Sec24p-cargo proteins targeting signals. (2.) Interaction between the lipids and transmembrane domains of cargo proteins. (3.) Treadmilling towards the ERES of the membrane bound COPII-cargo complex due to the asymmetrical recruitment of COPII on the ER side.

P417

Board Number: B559

Mutation in GAPDH that blocks endocytosis also arrests ER to Golgi transport.

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Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is required for ER to Golgi transport. GAPDH is also required for transport in the endocytic pathway; substitution of Pro236 to Ser rendered CHO cells defective in endocytosis. The objective of these studies was to determine if GAPDH (Pro236Ser) could inhibit ER to Golgi trafficking. To address this question, the recombinant mutant protein was evaluated in an in vitro trafficking assay. We found that the mutant protein efficiently blocked VSV-G protein transport suggesting that GAPDH (Pro236Ser) affected a factor(s) common to endocytosis and secretion. Permeabilized HeLa cells either incubated with mutant protein or ectopically expressing the mutant had fragmented Golgi. Moreover, Rab1 distribution was drastically altered in mutant treated/ectopically expressing cells, as assessed by indirect immunofluorescence. Immunoprecipitation and subsequent western blot analysis of cell lysates from wt and mutant transfected cells indicated that Rab1 associates with GAPDH but does not bind GAPDH (Pro236Ser). Importantly, the Pro236Ser mutation in GAPDH disrupted association between the enzyme and Akt. We propose that Rab1 requires GAPDH-Akt for ER-Golgi transport.

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P418**Board Number: B560****Sar1b loss-of-function animal model explains diverse physiological roles of chylomicron traffic in morphogenesis.**E.W. Knapik¹, D.S. Levic², W. Wang¹, D.B. Melville¹;¹Medicine, Vanderbilt University Medical Center, Nashville, TN, ²Cell and Developmental Biology, Vanderbilt University Medical Center, Nashville, TN

Anderson Disease (ANDD) or Chylomicron Retention Disease (CMRD) is a rare, hereditary lipid malabsorption syndrome associated with mutations in the SAR1B gene that is characterized by failure to thrive and hypocholesterolemia. Although the role of SAR1B in formation of coat protein II (COPII) coated carriers is well established, little is known about SAR1B loss-of-function in lipid absorption in vivo. Moreover, key questions regarding the pathogenesis of ANDD remain unanswered. To address this, we have developed a zebrafish model of Sar1b deficiency. Using pulse-chase lipid clearance assays and electron microscopy, we find that dietary fats accumulate in Sar1b-deficient enterocytes, consistent with ANDD symptoms of chylomicron retention. We also use in vivo imaging of dietary cholesterol analogs to reveal a novel requirement for Sar1b function in cholesterol uptake into enterocytes. ANDD patients present with a range of diverse symptoms, the pathogenesis of which is not known. Our study offers a model where specific symptoms and phenotypes can be tested. Using transgenic labeling, histology, and in situ analyses we show that Sar1b is required for exocrine pancreas and liver differentiation and growth. Furthermore, we find abnormal differentiation and maturation of craniofacial cartilage associated with defects in procollagen II secretion, as well as the absence of select, neuroD-positive neurons of midbrain and hindbrain. Our model of Sar1b-deficiency helps to systematically dissect developmental function of Sar1b and to discover molecular and cellular mechanisms leading to organ-specific ANDD pathology.

P419**Board Number: B561****Mechanism Regulating Synchronous Collagen Expression and Trafficking during Stem Cell to Chondrocyte Differentiation.**G. Unlu¹, E.W. Knapik²;¹Vanderbilt University, Nashville, TN, ²Medicine, Vanderbilt University Medical Center, Nashville, TN

Transition from the stem cell phase to a differentiated cell type is characterized by retooling cellular machinery, including regulatory molecules, structural proteins, and their transport machinery. To understand how the differentiation process provisions for synchronized changes in cellular functions during development, we used a robust in vivo model of zebrafish neural crest stem cells, which differentiate into chondrocytes and then produce, transport, and secrete procollagen II.

Although procollagen synthesis and trafficking are well studied, little is known about how collagen-specific transport machinery and collagen cargo are made synchronously available during vertebrate development and what mechanisms restrain this process from occurring in stem cells.

We have previously shown that procollagen transport requires COPII-inner coat components sec23a and Sec24D to promote collagen secretion (1,2), and a transcription factor, Creb3L2 to activate the expression of these COPII adaptors (3). We hypothesize that a stem cell factor temporarily restricts both collagen expression and trafficking machinery in the undifferentiated stem cell state; and its downregulation during chondrogenic differentiation allows for synchronous upregulation of collagen expression and secretion programs. Our in silico analyses suggested that a neural crest stem cell factor may bind to the promoter region of Creb3L2 to suppress collagen secretion as well as the promoter of a major chondrogenic factor Sox9 to inhibit collagen expression during cartilage development.

Using in vivo mosaic analysis, we find that overexpression of the stem cell factor in differentiated zebrafish chondrocytes and human fibroblasts downregulates both collagen expression and secretion. We demonstrate that overexpression of the stem cell factor leads to reduction in CREB3L2 promoter activity as detected by luciferase-based assays as well as decreased transcript levels of CREB3L2 and components of ER-to-Golgi (COPII) trafficking machinery, especially SEC23A and SEC24D. The changes at the transcript levels are matched by intracellular accumulation of type-I and type-II collagens. Our data support a model in which a neural crest stem cell factor acts as a master regulator of collagen expression and intracellular trafficking machinery; and its developmentally driven downregulation leads to synchronous upregulation of collagen cargo and its transport machinery during differentiation

References: (1) Lang, et.al., Nature Genetics 2006. (2) Sarmah, et.al., PLoS One, 2010. (3) Melville, et.al., Disease Models and Mechanisms, 2011.

P420

Board Number: B562

SLY41 Suppresses the Loss of YPT1 through Regulation of Intracellular Calcium.

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Newly synthesized secretory proteins and lipids are transported from the ER to the Golgi apparatus in a COPII-dependent pathway. This process can be separated into biochemically distinct stages:- of vesicle budding, tethering of vesicle to cis-Golgi membranes and ultimately SNARE-protein dependent membrane fusion. Genetic and biochemical approaches have been employed to identify the factors and characterize the mechanisms of each distinct stage. For vesicle tethering to Golgi membranes, the Rab GTPase Ypt1 functions by recruiting protein complexes important for vesicle recognition and tethering. Loss of *YPT1* gene function in yeast is lethal although a screen for suppressors of loss of *YPT1* identified a set of *SLY* genes that when overexpressed supported growth of *ypt1Δ* cells. Most of the *SLY* genes encode SNARE proteins or SNARE regulatory proteins that are thought to activate fusion and bypass the

Ypt1-dependent tethering stage. In contrast, *SLY41* encodes a multispanning membrane protein of the solute carrier (SLC) family and has no known function. In this study we characterize Sly41 and investigate the mechanism by which over-expression suppresses loss of Ypt1 function. Our findings show that Sly41 is efficiently packaged into COPII vesicles and actively cycles between the ER and Golgi compartments. A novel synthetic genetic interaction was identified between *SLY41* and the P-type ATPase Ca^{2+} transporter encoded by *PMR1*. Using aequorin-based techniques and inductively coupled plasma mass spectrometry, we detected elevated levels of intracellular Ca^{2+} in strains overexpressing Sly41. This effect of calcium is consistent with results showing that deletion of *PMR1* elevates intracellular Ca^{2+} levels and suppresses loss of Ypt1. Based on our data we are testing the hypothesis that Sly41 transports divalent cations such as Ca^{2+} and that overexpression increases intracellular Ca^{2+} levels to suppress tethering defects through activation of SNARE-dependent fusion of COPII vesicles with Golgi acceptor membranes.

P421

Board Number: B563

Fast live super-resolution imaging of a synchronous Golgi-to-ER transport wave.

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COPI vesicles mediate Golgi-to-ER retrograde transport. However, light microscopy has until recently been restricted to diffraction-limited structures (greater than ≈ 200 nm), making it impossible to follow COPI vesicles budding due to their small size and vicinity to the Golgi membranes. Here we generated a wave of COPI retrograde vesicles by recruiting a FKBP-KDEL (known to be contained in retrograde COPI vesicles) on a cis-Golgi localized FRB-tagged enzyme (ManII-FRB), taking advantage of the drug-induced FRB/FKBP dimerization system. We followed this wave of retrograde transport by Stimulated Emission Depletion (STED) microscopy on a custom built system, with lateral resolution better than 50 nm. Complete redistribution of ManII to the endoplasmic reticulum (ER) occurred within 1 hour after addition of the dimerizing drug. At earlier time points (10-30 mins) ManII-FRB/FKBP-KDEL dimers were incorporated into COPI-positive tubular-vesicular structures, which subsequently budded from the cis-Golgi side. Visualization of COPI vesicles budding from the Golgi in live cells at the nanoscopy level promises further insights in the mechanisms of formation of COPI in an intact living system.

P422**Board Number: B564****A role for a conserved domain within the Sec7 domain Arf guanine nucleotide exchange factor GBF1 in regulating dimerization, localization and function.**T. Busby¹, J. Bhatt¹, E.G. Viktorova², L. Newman³, G.A. Belov², R.A. Kahn^{3,4}, E. Sztul¹;¹Department of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, Birmingham, AL, ²Department of Veterinary Medicine, Virginia-Maryland College of Veterinary Medicine, University of Maryland, College Park, MD, ³Department of Biochemistry, Emory University School of Medicine, Atlanta, GA, ⁴Emory University School of Medicine, Atlanta, GA

Members of the large Sec7 domain Arf guanine nucleotide exchange factors (GEFs) appear to dimerize through their N-terminal DCB (dimerization and cyclophilin-binding) and HUS (homology upstream of Sec7) domains. However, the importance of dimerization for GEF function has not been assessed. In GBF1, a GEF functional at the ER-Golgi interface, the dimerization has been proposed to require the K91 and E130 residues within the DCB domain. Thus, we substituted both residues with alanines to generate a K91A/E130A (91/130) mutant and assessed its oligomerization status and function. We show that 91/130 is monomeric, that it targets to the Golgi in a manner indistinguishable from the wild-type dimeric GBF1, and that it rapidly exchanges between the cytosolic and membrane-bound pools. The monomeric 91/130 appears catalytically active as it integrates within the functional network at the Golgi, supports ARF activation and COPI recruitment, and sustains Golgi homeostasis and secretion. However, dimerization appears to stabilize GBF1 in cells and the 91/130 monomer is degraded faster than the dimeric GBF1. Our data support a model in which dimerization is not a key regulator of GBF1 catalytic activity, but impacts its function by regulating GBF1 cellular levels.

P423**Board Number: B565****Structure-function investigation of the C-terminal HDS domains of the Sec7 Guanine nucleotide exchange factor GBF1.**J.M. Bhatt¹, M. Mays¹, N.E. Kaushik¹, E. Sztul¹;¹Department of Cell, Developmental and Integrative Biology, University of Alabama at Birmingham, Birmingham, AL

Monomeric GTPases called ADP-ribosylation factors (ARFs) are critical for vesicular traffic. ARFs need to be activated by guanine nucleotide exchange factors (GEFs) that catalyze the GDP/GTP conversion on ARFs. Formation of COPI vesicles at the ER-Golgi interface has been shown to require the GEF GBF1. GBF1 is a multidomain protein consisting of a central Sec7 domain that catalyzes ARF activation and additional highly conserved domains with poorly understood functions. We hypothesized that the non-catalytic domains also play a role in GBF1 activity, and herein focus on the role of the HDS3 domain in modulating GBF1 function. Using multi-species and multi-member alignments of GBF1 orthologues we

identified a number of most highly conserved amino acids within the HDS3 domain and generated alanine replacements mutants: PLL1544AAA (PLL) and FPL1593AAA (FPL). Both mutants targeted to the Golgi, albeit with decreased efficiency. We assessed the role of the PLL and FPL motifs in "replacement assays" in which cells depleted of endogenous GBF1 by RNAi are 'replaced' with mutant GBF1. We show that the PLL and the FPL mutants are unable to support de novo Golgi synthesis as compared to wild-type GBF1. We investigated the folding of the proteins by limited proteolysis and found that the HDS3 mutants have altered folding patterns than wild-type GBF1. Our results suggest that the HDS3 domain plays a key role in the localization, folding and/or function of GBF1. Our future experiments are addressing two important questions: (1) what does the HDS3 domain interact with? And, (2) do the PLL and FPL mutations disrupt important GBF1 protein-protein interactions?

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Board Number: B566

Regulation of the cis-Golgi Arf-GEFs Gea1 and Gea2 by their HDS3 domains and the Rab GTPase Ypt1.

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The Golgi Arf GTPases control the sorting of membranes and proteins through all eukaryotic cells, recruiting adapters and cargo to sites of vesicle formation when in their activated GTP-bound form. Arf guanine nucleotide exchange factors (Arf-GEFs) are the master regulators of Arf activation and are conserved throughout eukaryotes. Two Arf-GEFs, Gea1 and Gea2, function at the *cis*-Golgi in *Saccharomyces cerevisiae*, but we know relatively little about how these GEFs are regulated. Recent work with their close relative at the *trans*-Golgi network, Sec7, has revealed that recruitment of Sec7 to the trans-Golgi network and relief of its autoinhibition are dependent on its C-terminal domains, as well as on positive feedback from Arf1 and other protein interactions. The Golgi Arf-GEFs share predicted domain architecture, suggesting that Gea1/2 may be regulated by mechanisms similar to those of Sec7. However, the specific proteins which orchestrate the activation and localization of Sec7 do not appear to act upon Gea1/2. *In vitro* biochemical studies, genetics, and live cell microscopy have revealed two contributing factors in Gea1/2 regulation: an interaction with the Rab GTPase Ypt1 and the regulatory roles of one of the domains of Gea1/2. Combined with structural studies, these results will define general features of Golgi Arf-GEF regulation and localization, as well as features specific to Gea1 and Gea2, providing fundamental insight into how Arf1 is regulated at the Golgi.

P425**Board Number: B567****Analyzing Polarized Protein Sorting at the Trans Golgi Network.**A. Treyer¹, F. Lazaro-Dieguez², M. Pujato¹, A. Muesch²;¹Albert Einstein College of Medicine, Bronx, NY, ²Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, NY

Transporting epithelial cells, such as those of the kidney, lung, gut, and liver, are situated at an interface between two different physiological environments in the body. To function, these cells rely on the generation of surface polarity, which includes initial segregation and continuous occlusion of large apical and basolateral surface domains. The protein and lipid identities of these domains have to be actively maintained throughout the lifespan of the epithelia due to constant turnover of these components in cellular metabolism. Polarized protein sorting and targeting is largely understood to be the function of the trans-Golgi network (TGN). Multiple studies in Mardin-Darby Canine Kidney (MDCK) cell lines, which both, exhibit polarized trafficking of apical and basolateral proteins, and are highly amenable to experimental manipulation, as well as in several other models have resulted in varying interpretations on how apical protein carriers are generated and on what basis are apical proteins segregated and targeted away from their basolateral counterparts. Since the TGN is a highly dynamic tubulo-vesicular structure, where the resident proteins are themselves subject to cargo-like cycling, no clear way exists to define this continuously transient organelle. In our studies, we are attempting to describe the properties of the TGN as a functional compartment by using temperature and pharmacological modulation of the secretory pathway to pulse chase fluorescent cargo to the TGN, while preventing its exit to the post-Golgi destinations, and thus attenuating the lifespan and function of the transient sorting domains. We have developed a fluorescence-based analysis protocol for membrane extracts that contain sorted cargo fractions, and we are proceeding to quantitatively describe the extent to which different cargo types become sorted in the TGN, as well as to further understand how the architecture of this compartment supports the observed sorting dynamics.

P426**Board Number: B568****PCAF is involved in acetylation and subcellular distribution of Sigma-1 receptors.**Y. Yasui¹, T. Su¹;¹NIDA, NIH, Baltimore, MD

Sigma-1 receptors (Sig-1Rs) are endoplasmic reticulum (ER) chaperon proteins that relate to a variety of cellular functions such as ion channel activities, ER-mitochondrial interorganellar calcium signaling, cellular survival and neuronal differentiation. Sig-1Rs predominantly reside at the ER subdomain-apposing the mitochondria (the mitochondria-associated ER membrane, MAM) and translocate to other compartments of the cell under certain conditions such as cellular stress and stimulation with the ligands including cocaine. We previously showed that cocaine-induced translocation of Sig-1Rs, which

intensifies the interaction between Sig-1Rs and Kv1.2 potassium channel, plays an important role in behavioral and neuronal responses to cocaine in mice. Thus, the dynamic shift of subcellular distribution is a critical step for Sig-1Rs to execute their functions at remote sites other than the MAM. However, the detailed mechanism on how the translocation is initiated is largely unknown.

In this study, we found that Sig-1Rs interact with a histone acetyltransferase, p300/CBP-associated factor (PCAF) and that cocaine affects the interaction. We found that the interaction between PCAF and Sig-1Rs occurs at the peri-nuclear area by using immunofluorescence studies. We also found that Sig-1Rs can be acetylated and the acetylation level is modulated by the overexpression or knockdown of PCAF. Protein acetylation on lysine residue(s) is known to usually play a key role in regulating protein functions that relate to nuclear and cytoplasmic events. However, recently it has been reported that acetylation of membrane proteins at the lumen of the ER regulates the protein translocation at the early secretory segment of the translocation pathway. Since Sig-1Rs have lysine residue(s) in the C-terminus that faces the lumen of the ER, we hypothesized that the acetylation of Sig-1Rs could be involved in Sig-1Rs' translocation. We therefore examined whether the PCAF expression level may affect Sig-1R's subcellular distribution by density gradient centrifugation. The results showed that overexpression or knockdown of PCAF changed the Sig-1Rs' subcellular distribution. We also confirmed that cocaine treatment changed the subcellular distribution of Sig-1Rs. However, the cocaine-induced distribution pattern of Sig-1Rs is not exactly the same as those caused by overexpression or knockdown of PCAF.

Together, our data show for the first time that PCAF is a molecular protein, which plays important roles in the acetylation and subcellular distribution of Sig-1Rs. Although cocaine affects the interaction between Sig-1Rs and PCAF, whether the PCAF-mediated acetylation of Sig-1Rs may fully explain cocaine-induced Sig-1R translocation remains to be clarified in the future.

P427

Board Number: B569

Cbl-c: A Regulator of Golgi Organisation.

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The Golgi apparatus plays a pivotal role in the secretory pathway through post-translational modification of secretory and membrane proteins and their subsequent sorting to appropriate destinations. Golgi organisation is dependent on a complex local signalling network. Src is a tyrosine kinase that controls Golgi structure and function. Src is regulated by various ubiquitin ligases, including Cullin-5 and Cbl family proteins. Here we find in a screen for Golgi regulators that Cbl-c depletion induces Golgi fragmentation, while c-Cbl, Cbl-b and Cullin-5 do not. This indicates Cbl-c is specifically involved in regulating Golgi organisation. Furthermore, the ubiquitin ligase activity of Cbl-c is required for its role in regulating Golgi organisation. Cbl-c protein is partially localised on Golgi membranes and this localisation is dependent on Src activity. We find that Cbl-c's regulation of Golgi organisation is dependent on Src, suggesting that Cbl-c regulates COPI dynamics. Cbl-c is self-ubiquitinated in the

presence of active Src. Overall, our results demonstrate a novel role of Cbl-c in regulating Golgi organisation.

P428

Board Number: B570

Initial characterization of non-ER or -Golgi related nanotubes in protein transport.

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As written in the textbook, most protein transport within the cell relies on vesicular transport, which utilizes either small and spherical vesicles, or large and irregularly-shaped membrane-enclosed compartments. Through a specific labeling approach, we are able to show that there are extensive amounts of nanometer-scale tube-like structures (nanotubes) in the cytosol, participating in protein transport. Unlike linear fragments of ER or Golgi apparatus align along microtubules, these nanotubes have limited co-localization with either dynamic or stable populations of microtubules. Interestingly, some but not all of these nanotubes are regulated by dynamic actin filaments. Under stress conditions, the amount of nanotubes increases over time. It would be intriguing to know how these non-ER or -Golgi related nanotubes are generated, and why they exist. We postulate that these nanotubes function as tunnels to expedite protein transport.

P429

Board Number: B571

BI-1 regulates insulin secretion through enhanced proinsulin synthesis and Glut2 glycosylation in ER.

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Type 2 diabetes mellitus (T2DM) is a metabolic disorder in which pancreatic insulin secretion does not meet the demands of insulin sensitivity. It is now accepted that elevated glucose levels are required to mediate the lipotoxic effects, including inhibition of glucose-stimulated insulin secretion (GSIS). Chronic hyperglycemia has been known to impair beta cell function. Glucose transporters (Gluts) are a family of integral membrane glycoproteins that transport saccharides across the cell plasma membrane by facilitative diffusion to supply metabolic energy. Pancreatic beta cell-surface expression of glucose transporter-2 (Glut-2) is essential for glucose-stimulated insulin secretion (GSIS), thereby controlling blood glucose homeostasis. N-glycosylation of Glut-2 is required for Glut-2 residency. In this study, Bax inhibitor -1 (BI-1) enhances Glut-2 expression on the β cell surface by regulating N-glycan

ligand for pancreatic lectin receptors. BI-1 enhanced Glut-2 cell surface half-life. The impairment of GSIS leads to metabolic dysfunction diagnostic of type 2 diabetes. Remarkably, the induction of diabetes by chronic ingestion of a high-fat diet is associated with attenuated Glut-2 glycosylation. Chronic hyperglycemia-induced insulin secretion impairment in human insulinoma (INS-1) cells and pancreatic beta islet cells, is regulated by BI-1. BI-1 highly maintains proinsulin synthesis in ER. In the presence of BI-1 insulin secretion is triggered by glucose after chronic hyperglycemia, most likely by enhanced glucose uptake through glycosylation. Ex vivo and in vivo study, we found a correlation between post-translational glycosylation of the Glut-2 and its associated chaperone, calnexin and calreticulin. These results indicate that BI-1 has a regulatory role in diabetes mellitus and can be a therapeutic target protein.

P430

Board Number: B572

The COPI vesicle coat interacts with the Dsl1 tethering complex at polarized ER arrival sites.

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Retrograde trafficking from the Golgi apparatus to the endoplasmic reticulum (ER) is carried out by COPI vesicles. In order to fuse with the ER membrane, COPI vesicles must at least partially disassemble their coat to expose membrane and fusion machinery. However, to date it is still not known during which transport step the coat is removed, and which factors regulate the removal. The ER-resident Dsl1 tethering complex of *Saccharomyces cerevisiae* has recently been implicated in this process, as it was shown to interact with COPI coat components *in vitro*. Here, we provide *in vivo* evidence for the interactions between Dsl1 complex and COPI coat and cargo proteins under native expression conditions, using a bimolecular fluorescence complementation (BiFC) approach. By employing the BiFC system as a unique tool for *in vivo* crosslinking, we visualize the otherwise transient interactions between COPI vesicles and Dsl1 complex, and show that the interactions are substantial for cell viability. Our results carry two interesting implications: They provide evidence for COPI coat retention until arrival at the ER membrane, suggesting a targeting function of the coat complex. These results are complemented by our reports of genetic interactions between the Dsl1 complex and a set of p24 cargo receptors, which may in this context fulfill an additional regulatory role in vesicle uncoating. Furthermore, the visual detection of interaction sites between Dsl1 complex and COPI coat at the ER unveils the subcellular localization of ER arrival sites in *S. cerevisiae*. The ER arrival sites show a distinct localization with respect to ER exit sites, Golgi, and sites of secretion, and exhibit a growth-associated polarization pattern. These dynamics provide insights into the crosstalk between transport processes, suggest a function of COPI-mediated retrograde transport in cell budding, and conceivably play a role in organelle inheritance.

P431**Board Number: B573****Sac1 Phosphoinositide Phosphatase – Regulation of PI(4)P levels in the early secretory pathway.**S. Weiss¹, C. Barlowe¹;¹Biochemistry, Geisel School of Medicine, Dartmouth College, Hanover, NH

Phosphoinositides play multiple roles in intracellular trafficking and can act as intracellular signals, which recruit proteins to specific compartments. The restricted distribution of phosphoinositides is created by targeting of lipid modifying enzymes. PI(4)P is synthesized by multiple, separate PI(4)P-kinases and is therefore likely to have distinct intracellular pools. A previous study from our lab has shown that inhibition of PI(4)P levels on Golgi membranes results in decreased ER-to-Golgi transport efficiency (Lorente-Rodriguez and Barlowe, 2010). Based on these results, we proposed that Golgi-localized PI(4)P regulates fusion of COPII vesicles with Golgi membranes.

In this study, we investigate the role of the phosphoinositide phosphatase Sac1 in regulation of PI(4)P levels at *cis*- and *medial*-Golgi compartments. Localization of Sac1 was determined by sucrose gradient fractionation and live cell imaging experiments and compared to Sac1 localization under glucose starvation conditions. Endogenous Sac1 as well as Sac1-GFP remained ER-localized in the presence or absence of glucose starvation. In cell-free COPII-dependent vesicle budding assays we observed that Sac1 was not efficiently packaged into COPII vesicles. These results support a model in which Sac1 is ER-localized and fulfills its role in regulation of different PI(4)P pools through contact sites between the ER and other cellular compartments such as the Golgi or the plasma membrane. To understand which PI(4)P pools are targeted by ER-localized Sac1, we constructed different versions of the Sac1 protein. Overexpression of a soluble Sac1 protein, which lacks the two transmembrane domains (Sac1 Δ TM), rescues the cold sensitivity of *sac1* Δ cells, whereas the phosphatase dead mutants Sac1 C392S or Sac1 Δ TM C392S failed to rescue cold sensitivity. Furthermore, we observed that the Sac1 Δ TM C392S protein, which shows high affinity to PI(4)P but lacks the phosphatase activity was targeted to membranes compared to the soluble Sac1 Δ TM protein. GFP-tagged versions of Sac1 Δ TM C392S provide a new, powerful tool to analyze Sac1 function and intracellular localization in more detail.

P432**Board Number: B574****Congenital dyserythropoietic anemia type II reveals an interaction of the COPII coat component SEC23 with KIF23 and integration of cell cycle progression with membrane traffic.**F. Verissimo¹, k. schwarz², M. Gentzel³, R. Pepperkok⁴;¹EMBL, Heidelberg, Germany, ²Institute for Transfusion Medicine, University of Ulm and Institute of Clinical, Ulm, Germany, ³Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany,⁴Cell Biology and Biophysics, European Molecular Biology Laboratory (EMBL), Heidelberg, Germany

Congenital dyserythropoietic anemia type II (CDAlI) is caused by mutations in the Sec23b gene. Yet it's molecular mechanism and how mutations that span the complete gene cause the disease remains elusive. Here we propose that CDAlI is caused by the failure of the interaction of SEC23 with the mitotic regulator KIF23/MKLP1. The localization of these proteins at the mitotic spindle in metaphase is necessary for recruitment of KIF23 downstream effectors and successful completion of cell division. On the other hand localization of SEC23 to the mitotic spindle avoids its degradation and guarantees sufficient SEC23 for the reformation of the secretory pathway at the end of mitosis. Altogether, our results uncover a new role for the SEC23 protein in cell division, provide a mechanistic explanation for the congenital dyserythropoietic anemia type II (CDAlI) and describe a novel mechanism that integrates cell cycle progression with regulation of the early secretory pathway.

P433**Board Number: B575****Contact of cis-Golgi with ER exit sites executes cargo capture and delivery from the ER.**K. Kurokawa¹, A. Nakano²;¹Center for Advanced Photonics, RIKEN, Wako, Japan, ²Center for Advanced Photonics, RIKEN, Wako-shi, Japan

Protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus is mediated by coat complex II (COPII) vesicles. Sar1-GTP recruits the inner shell complex (Sec23/24) to initiate COPII coat assembly and sorting of secretory cargo proteins, and then the outer cage complex (Sec13/31) promotes the polymerization of the whole coat and deforms ER membrane to drive COPII vesicle formation at the ER exit sites (ERES). It has been believed that COPII vesicles containing cargo are released from ERES into the cytosol and then reach and fuse with the first post-ER compartment, cis-Golgi or ER-to-Golgi intermediate compartment (ERGIC). However, it still remains elusive how cargo loading to vesicles, vesicle budding, tethering and fusion are coordinated in vivo. Here we show, using extremely high speed and high resolution confocal microscopy (which is named as Super-resolution Confocal Live Imaging Microscopy, SCLIM), that the cis-Golgi in the budding yeast *Saccharomyces cerevisiae* approaches and

contacts the ERES. The COPII coat cage then collapses, and the cis-Golgi captures cargo. The cis-Golgi, thus loaded with cargo, then leaves the ERES. We propose that this “hug-and-kiss” behavior of cis-Golgi ensures efficient and targeted cargo transport from the ERES to cis-Golgi.

P434

Board Number: B576

Super-resolution imaging reveals anterograde and retrograde cargos in COPI vesicles.

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COPI-coated vesicles play a central role in the secretory pathway. They have been strongly implicated in both anterograde and retrograde transport, though their role in anterograde transport remains controversial. COPI vesicles are approximately 70 nm in diameter and closely packed around the Golgi, which prohibits direct visualization of individual vesicles, or their cargo, with conventional light microscopy (limited to 250 nm resolution).

Recent advances in stimulated emission depletion (STED) and single molecule switching (SMS) super-resolution microscopy approaches now enable the examination of individual COPI vesicles. We have accordingly developed techniques to label endogenous proteins in COPI vesicles and use super-resolution microscopy to identify individual COPI vesicles in fixed cells. Using two and three-color imaging, we have determined that COPI vesicles contain the expected protein machinery such as the scaffold Scyl1 and the v-SNARE Bet1, while lacking Golgi cisternal tether proteins GM130 and GRASP65. We also confirm that COPI vesicles contain both anterograde and retrograde cargos (Orci *et al.* 1997, Cell 90: 335-349).

P435

Board Number: B577

All roads lead to Rome- SRP independent translocation into the endoplasmic reticulum.

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Translocation into the endoplasmic reticulum (ER) is an initial and crucial biogenesis step for all secreted and endomembrane proteins in eukaryotes. Even in the simple eukaryotic model organism, *Saccharomyces cerevisiae*, this is no simple task, as over a fifth of its proteome must translocate into the ER. It's been well established that several ER targeting pathways are present in *S. cerevisiae*, the best known of which relies on the signal recognition particle (SRP). We set out to shed new light on

alternative SRP-independent translocation pathways. To do so, we harnessed unbiased and systematic approaches to further our understanding of the mechanisms by which these pathways function, and what measures are in place if they are dysfunctional. By combining hydropathy-based analysis and high throughput microscopy, we uncovered that over 20% of the yeast secretome translocates without the aid of the SRP. Further investigation of these SRP-independent substrates revealed an additional motif for ER targeting and uncovered a network of cytosolic proteins that facilitate SRP-independent targeting and translocation. Finally, by employing a systematic microscopic screen, we revealed that SRP-independent substrates are subject to pre-translocational monitoring that clears the cytosol of proteins that have failed to translocate in a timely manner. These findings highlight the underappreciated complexity of SRP independent translocation and its central role in enabling the extensive flux of proteins into the ER.

Voltage-Gated Channels and Neurotransmission

P436

Board Number: B579

Kismet affects synaptic transmission and endocytosis at the *Drosophila* Neuromuscular Junction.

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Efficient synaptic communication requires bidirectional signaling between the pre- and postsynaptic cells. This signaling depends on the localization of synaptic proteins to mediate endo- and exocytosis at appropriate synaptic locations. We have found that the trithorax group chromodomain helicase DNA binding (CHD) protein, Kismet (Kis), which regulates chromatin remodeling, influences synaptic transmission and, possibly secondarily, endocytosis. Animals with mutations in *kis* exhibit a significant decrease in the frequency and amplitude of spontaneous neurotransmission and in evoked amplitudes. The change in neurotransmission may be partly due to a reduction in endocytosis in *kis* mutants. Further, *kis* mutants exhibit a change in the localization of the postsynaptic glutamate receptor subunit, GluRIIC, relative to Brp, a protein localized to active zones. Our data suggests that Kis affects synaptic transmission and endocytosis by regulating distinct processes in both the pre- and postsynaptic cells. Based on these data, we hypothesize that Kis regulates synaptic transmission, possibly by influencing transcription of synaptic target genes involved in bidirectional synaptic signaling and/or endocytosis.

P437**Board Number: B580****Regeneration of Olfactory Epithelium from Horizontal Basal Cells.**R.E. Turn¹, C. Wall², H. Zhao²;¹Harriet L. Wilkes Honors Coll, Jupiter, FL, ²Department of Biology, Johns Hopkins University, Baltimore, MD

Due to environmental insult and natural turnover, olfactory neurons and the surrounding non-neural cells die and therefore need to be replaced to restore function. Two forms of progenitor cells facilitate the regeneration of neural tissue within the pseudostratified olfactory epithelium: globose basal cells (GBCs) and horizontal basal cells (HBCs). While GBCs regularly produce progeny which mature into olfactory neurons, HBCs only undergo mass regeneration after widespread loss of olfactory epithelium. HBCs are capable of producing all olfactory epithelium cell types. Furthermore, these pluripotent progenitor cells are found in adult mammals and are easily accessible via biopsy. These definitive characteristics of HBCs make them a promising population of adult neural stem cells that may be used for future medicinal applications. As a result, there is a push to better characterize HBCs and the mechanisms they employ to restore neural and non-neural tissue post-injury.

Recently, our lab has investigated different ways to activate HBCs and to characterize signaling after activation. To do so, we designed a Cre/LoxP mouse line that expresses the fluorescent marker protein, TdTomato, in members of the HBC lineage. Quantification of fluorescent and non-fluorescent cells in the olfactory epithelium was conducted subsequent to chemically-induced damage via intraperitoneal injections of the drug methimazole. We wish to define the relationship between administered dosage and resulting damage using flow cytometry, to identify the extent of regeneration after different modes of damage and recovery times, and to characterize HBC lineage protein expression at the time of activation (1-2 days post injury).

P438**Board Number: B581****Extracellular stimuli regulate cell differentiation and acquisition of functional properties in cultured embryonic chick olfactory neurons.**G. O'Neill¹, C. Musto¹, G. Gomez¹;¹Biology, University of Scranton, Scranton, PA

Cell differentiation is often governed by endogenous compounds, such as growth factors. However, sensory systems such as olfaction are unique because they are constantly exposed to external compounds as part of their normal function. Thus, our aim was to test if these external stimuli can exert similar effects on the differentiation of sensory cells. Olfactory turbinates were extracted from E-17 chick embryos, enzymatically dissociated, then grown on glass coverslips. Starting on day 2, cells were exposed to different odors for 12 hour periods, alternated with periods of no-odor exposure. On days 4,

5, and 6, cell odor sensitivity was tested using calcium imaging, and the expression of proteins characteristic of neuronal differentiation was quantified using immunocytochemistry. Our results show that a greater proportion of cells exposed to odors are odorant responsive. In addition, immunocytochemistry results showed that a higher proportion of treated cells were beta-tubulin+ and OMP+. Odor stimuli canonically activate signal transduction cascades mediated by cAMP. Our studies suggest multiple long-term roles of these signal transduction cascades that are traditionally thought to activate short-term signaling activities. This opens avenues for future investigations on the role of normal extracellular environments on directing neural cell development.

P439

Board Number: B582

Uncovering a restrictive molecular mechanism that regulates dendritic spine formation in the developing brain.

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Within the brain, information is relayed across pathways of excitatory synapses. These excitatory synapses individually localize at specialized cellular compartments on neuronal dendrites called dendritic spines. Although dendritic spines are independently remodeled over an individual's lifetime, certain parameters of dendritic spine development are stereotyped in the early postnatal brain, including the locations, timing, and density of nascent spine formation. The restrictive molecular mechanisms defining these parameters to prevent aberrant dendritic spine formation are unknown.

The RhoA GEF Ephexin5 is a negative regulator of dendritic spine development that is ubiquitinated and degraded during events preceding dendritic spine formation. Ephexin5 localizes to dendritic spines and drives significant RhoA activation in the early brain, and I hypothesize based on this activity that the restrictive role of Ephexin5 is regulated by mechanisms enforcing where, when, and how often dendritic spines form during development.

By monitoring Ephexin5 expression over time, we have discovered that Ephexin5 is phosphorylated at N-terminal serines 107 and 109 during a developmental time course preceding synaptogenesis. This phosphorylation is required for Ephexin5-mediated RhoA activation and dendritic spine suppression in cultured neurons. Furthermore, Ephexin5 serine 107 and 109 phosphorylation appears to be mediated by a PKC isozyme that we are currently pursuing. The identification and characterization of Ephexin5-targeted kinase activity will ascribe a broadened role to the responsible enzyme in neuronal development, meanwhile enabling us to elucidate a restrictive molecular mechanism that parameterizes dendritic spine formation in the early postnatal brain.

P440**Board Number: B583****Transsynaptic protein alignment organized by a synaptic nanocolumn.**A. Tang¹, T.A. Blanpied^{2,3};¹Physiology, University of Maryland School of Medicine, Baltimore, MD, ²Department of Physiology, Univ of Maryland Sch of Med, Baltimore, MD, ³Program in Neuroscience, Univ of Maryland Sch of Med, Baltimore, MD

Accumulation of neurotransmitter receptors across from sites of synaptic vesicle fusion is the structural basis for synaptic transmission, and the molecular aspects of this organization are of great interest. Key critical elements are abundant and essential scaffold proteins that establish the architecture of the PSD; deletion or mutations in their human genes cause severe neuropsychiatric disorders including autism, mental retardation, and schizophrenia. Previously, by measuring the internal structure of single PSDs in live neurons using photoactivated localization microscopy (PALM), we have found that four major PSD scaffold proteins, PSD-95, GKAP, Shank and Homer1, were each organized in distinctive ~80 nm ensembles. Notably, PSD-95 ensembles were enriched for both GluA2-containing AMPARs and GluN2B-containing NMDARs. The functional influence of such an organization is predicted to be greatly amplified if postsynaptic receptor/scaffold ensembles lie very precisely across the synaptic cleft from sites of vesicle fusion within the active zone. However, whether such alignment exists has not been demonstrated.

Here, we used multiple-color 3D stochastic optical reconstruction microscopy (STORM) to examine the distribution of other synaptic constituents, including presynaptic proteins known to be involved in mediating vesicle fusion. Among these, RIM, a protein thought to link vesicles with Ca channels, was found organized in high-density ensembles within single active zones. These ensembles were of size similar to postsynaptic scaffold and receptor nanoclusters (76 ± 9 nm diameter). Critically, cross-correlation analysis showed that RIM nano-ensembles were highly correlated to the position of PSD-95. Furthermore, this correlation was reduced by picrotoxin treatment that enhances neuronal activity and is known to homeostatically weaken synaptic transmission. These results indicate that multiple proteins distributed along the axial extent of the synapse are co-enriched at specific domains across the face of the synapse. Further, this transsynaptic alignment is modulated by activity. This relatively compact, vertically oriented molecular organization within the bounds of the synapse suggests a “synaptic nanocolumn” that may be important for establishing and modulating synapse function.

P441**Board Number: B584****Distinct spatial distributions of evoked and spontaneous vesicle fusion sites.**H. Chen^{1,2,3}, T.A. Blanpied^{1,2},¹Program in Neuroscience, Univ of Maryland Sch of Med, Baltimore, MD, ²Department of Physiology, Univ of Maryland Sch of Med, Baltimore, MD, ³Medical Scientist Training Program, Univ of Maryland Sch of Med, Baltimore, MD

Healthy synapse function relies on precise structural alignment between the presynaptic active zone (AZ) and the postsynaptic density (PSD). A central question is whether localization of vesicle fusion sites influences the efficacy of synaptic transmission. The question is important because receptor activation at glutamatergic synapses is limited by both biophysical properties of the receptors themselves and their distance from vesicle release sites. Thus, release events can fail to activate all synaptic receptors. Recently, our lab found that PSD scaffolding proteins cluster receptors into nanometer-scale subregions. This organization is expected to increase the impact of fusion site organization: simulations show that EPSC amplitude at synapses with clustered receptor distributions is greatest when vesicle fusion is aligned with postsynaptic clusters. However, it has not been previously possible to precisely localize vesicle fusion sites, and so there is little information about where within the AZ vesicles fuse. The significance of this issue is emphasized by the growing list of diseases in which cognitive and behavioral deficits appear to stem from disruption of synapse function caused by mutations of genes such as RIM1 and Munc13 that encode synaptic proteins. To map vesicle fusion sites, I developed a novel technique to localize single-vesicle fusion with high spatial resolution, which I call "pHluorin uncovering sites of exocytosis" or pHuse. Using this approach, I mapped the pattern of evoked or spontaneous vesicle fusion at individual presynaptic terminals of cultured hippocampal neurons. Spontaneous release of neurotransmitter was previously thought to be biological noise but has recently been linked to distinct physiological functions. Interestingly, there is controversy over whether spontaneous and evoked release utilize different vesicle pools, involve different trafficking and fusion machinery, or activate different groups of receptors. All these factors suggest that spontaneous and evoked fusion could take place at spatially distinct regions of the AZ, but this has not been tested. My data using pHuse indicate that evoked and spontaneous fusion in fact occur over different subregions of the terminal. Specifically, when normalized to bouton area, we found that evoked fusion occurred over a significantly smaller proportion of the terminal (0.13 ± 0.02) than spontaneous fusion (0.20 ± 0.02 , Kolmogorov-Smirnov (K-S) test, $D=0.26$, $p < 0.01$). Thus, we propose that the spatial distributions of evoked and spontaneous vesicle fusion are differentially regulated by specific active zone proteins and activity. In sum, these data will help elucidate the organization and regulation of a key aspect of synaptic function.

P442**Board Number: B585****Disruption of Calcium Signaling Is Necessary for Ectopic Neuronal Cell Cycle Re-entry and Subsequent Neuron Death in Alzheimer's Disease.**E.J. Kodis¹, G.S. Bloom²;¹Department of Biology, University of Virginia, Charlottesville, VA, ²Departments of Biology and Cell Biology, University of Virginia, Charlottesville, VA

Alzheimer's disease (AD) is a neurological disorder characterized histologically by two types of lesions in the brain—extracellular plaques composed of amyloid- β ($A\beta$) peptides, and neurofibrillary tangles composed of the protein, tau. It has been demonstrated that $A\beta$ and tau aggregates have toxic effects on neurons, but less is understood about how the interaction of these two molecules leads to neuron dysfunction and death. $A\beta$ peptides act upstream of tau in AD pathogenesis, and it has been demonstrated that $A\beta$ peptides interact with multiple proteins and receptors at the cell surface. In particular, $A\beta$ oligomers ($A\beta$ Os) have been shown to bind and cause calcium influx through the N-methyl-D- aspartate (NMDA) receptor, increasing steady-state calcium levels and contributing to excitotoxicity in neurons vulnerable in AD. Although aberrant calcium influx and excitotoxicity due to $A\beta$ O exposure is well established, there is still little known about the downstream signaling consequences of how this calcium influx contributes to neuron death in AD. Neurons in adult brain are normally in a permanently post-mitotic state, but in AD they often exhibit ectopic cell cycle re-entry (CCR), which ironically leads to their eventual death. Our lab previously reported that neuronal CCR is initiated by $A\beta$ Os and requires tau (Seward, et al. J Cell Sci 126: 1278-1276). We now report that in addition to the NMDA receptor being an important mediator of excitotoxicity and synapse loss, it is also necessary for neuronal CCR. When primary neuron cultures are treated with $A\beta$ Os, they re-enter the cell cycle through G1 (as demonstrated by cyclin D1 expression) or S-phase (as demonstrated by nuclear BrdU incorporation), but treatment with the NMDA receptor inhibitor, MK-801, prevents CCR. Furthermore, $A\beta$ O treatment of primary neurons causes an early activation of CaMKII, a protein kinase known to be both dysregulated in AD and necessary for CCR. This $A\beta$ O-mediated CaMKII activation is prevented by shRNA knockdown of the NMDA receptor subunit NR1. This result suggests that $A\beta$ O toxicity at the synapse and excess calcium influx are directly connected to CCR and eventual neuron death in AD. Using this aberrant re-entry of post-mitotic neurons into the cell cycle as a read out of $A\beta$ O toxicity, signaling pathways connecting $A\beta$, tau, and calcium in AD are being further elucidated.

P443**Board Number: B586****Regulation of Low Voltage Activated Calcium Channels (Cav3) by Cyclin Dependent Kinase 5.**A. Calderon-Rivera¹, R. Gonzalez-Ramirez², A. Sandoval¹, R. Felix³;¹School of Medicine FES Iztacala, UNAM, Tlanepantla, Mexico, ²Department of Molecular Biology and Histocompatibility, Hospital General Dr. Manuel Gea González, Mexico City, Mexico, ³Department of Cell Biology, Cinvestav-IPN, Mexico City, Mexico

Low Voltage Activated (LVA) T-type calcium channels open in response to small membrane depolarization, and therefore represent an important source of calcium entry near the resting membrane potential. In neurons, these proteins significantly contribute to control relevant physiological functions including excitability, pacemaker activity and post-inhibitory rebound burst firing. Three subtypes of LVA channels (Ca_v3.1, Ca_v3.2 and Ca_v3.3) have been identified, and using functional expression of recombinant Ca_v3 channels diverse studies have validated the notion that T-type channels can be dynamically modulated by endogenous ligands as well as by second messenger pathways. In this context, the present study reveals a previously unrecognized role for cyclin-dependent kinase 5 (Cdk5) in the regulation of native T-type calcium channels in N1E-115 neuroblastoma cells as well as Ca_v3.1 channels heterologously expressed in HEK-293 cells. Cdk5 and its co-activators play critical roles in the regulation of neuronal differentiation, cortical lamination, neuronal cell migration and axon outgrowth. Our results show that overexpression of Cdk5 causes a significant increase in whole cell patch clamp currents through T-type calcium channels in N1E-115 cells, while knockdown of Cdk5 or the use of an inhibitor reduced T-type currents. Consistent with this, overexpression of Cdk5 in HEK-293 cells stably expressing Ca_v3.1 channels up-regulates macroscopic current. These results highlight a novel role for Cdk5 in the modulation of T-type calcium channels.

Anterograde and Retrograde Axonal Treatment**P444****Board Number: B587****Intra-Axonal Translational Control Mechanisms for ER Chaperone Protein mRNAs.**A. Pacheco¹, J.L. Twiss², G. Gallo³;¹Temple University, Philadelphia, PA, ²Dept of Biological Sciences, University South Carolina, Columbia, SC, ³Shriners Hospitals Pediatric Research Center, Temple University, Philadelphia, PA

Transport of mRNAs into distal nerve terminals and growth cones allows axonal processes to generate proteins autonomously from the cell body. Sensory neurons transport a complex population of mRNAs into their axons, including several encoding ER chaperone proteins. It remains largely unknown how

specificity is provided to the localized translational apparatus. Under cellular stress, protein synthesis can be regulated by both cap-dependent and cap-independent mechanisms (e.g., internal ribosome entry site [IRES]). We previously demonstrated that sensory axons have the capacity to translate mRNAs through IRES. For instance, axonal grp78/BiP but not calreticulin mRNAs' 5' untranslated regions [UTRs] have IRES activity. However, a simple binary choice between cap-dependent and cap-independent translation cannot explain the specificity for translation of individual mRNAs in distal axons. For calreticulin mRNA, one of its two 3' UTR elements can drive its localization to axons but its 5'UTR was shown to confer translational control through a mechanism that requires phosphorylation of eIF2 α in axons after stimulation of ER stress with LPA. This phosphorylation of eIF2 α typically blocks cap-dependent translation. Interestingly, the proximal 3'UTR localization element in calreticulin mRNA is essential for this phospho-eIF2 α -dependent translational control. Thus, 5' and 3' UTRs can perform concerted roles to achieve specificity for axonally localized mRNA translation. LPA is a physiological stress stimulus that induces growth cone collapse in DRG neurons and it is released after tissue injury. Since it has been shown that CNS inhibitory molecules can influence local translation in axons, we wondered if inhibitors molecules associated to injury response could regulate calreticulin expression. CSPGs (chondroitin sulfate proteoglycans) are extracellular matrix proteins produced by activated astrocytes in the injured CNS that stall the growth cone and forward movement of the axon. This effect extends to PNS sensory neurons that when plated onto substrates coated with CSPGs show extremely attenuated axonal outgrowth. We used immunostaining to detect axonal calreticulin of cultured DRG (dorsal root ganglion) neurons initially grown in CSPG coated coverslips. The results showed an increased signal in distal axons after 24h growing. Further experiments for local axonal translation regulation are being performed. We suggest that calreticulin mRNA elements could drive dual response to CSPGs vs other released stress signals after axonal injury.

P445

Board Number: B588

Cytoplasmic dynein overrides other motors to transport axonal microtubules in a manner that establishes and preserves their uniform pattern of polarity orientation.

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We have proposed that the forces generated by cytoplasmic dynein are essential for the axon to maintain a nearly uniform plus-end-distal polarity pattern of its microtubules. The premise is that cytoplasmic dynein populates the axon with correctly oriented microtubules by transporting them with their plus-ends-leading from the cell body into the axon and down its length. Just as important, if minus-end-distal microtubules arise in the axon, we propose that cytoplasmic dynein would transport them back to the cell body to prevent them from accumulating. Such mal-oriented microtubules may

arise during plastic events such as branch formation, or as a result of disease or injury-related challenges. While the majority of microtubule transport in the axon is anterograde, a notable fraction is retrograde, which is consistent with the existence of such a clearing mechanism. However, the role of cytoplasmic dynein has proven technically difficult to investigate because previous methods for inhibiting or depleting this motor protein were only able to do so gradually, thus introducing the potential for compensatory changes in the levels of other motor proteins. Here, we used Ciliobrevin D, a novel small molecule inhibitor, to acutely inhibit cytoplasmic dynein in cultures of rat sympathetic neurons. Exposure of the neurons to the drug for various windows of time resulted in a dose-dependent appearance of greater numbers of minus-end-distal microtubules in the axon. In addition, and consistent with our hypothesis, microtubule transport events were severely reduced in frequency in both the anterograde and retrograde directions. The microtubule movements that did occur in the presence of the drug exhibited abnormalities including abrupt pausing or halting of movements and directional changes in the movements not observed in control axons, as well as abnormal transport rates. These data suggest that a variety of molecular motors are able to transport microtubules in the axon, but that cytoplasmic dynein overpowers the others to ensure that microtubule transport occurs predominantly or exclusively with plus-ends-leading. We conclude that the microtubule polarity pattern of the axon requires constant maintenance against corruption, and that dynein-driven microtubule transport ensures that mal-oriented microtubules do not accumulate in the axon. Pathogenic situations that may overwhelm or compromise this mechanism could permit corruption of the microtubule polarity pattern of the axon sufficient to cause problems such as organelle traffic jams.

P446

Board Number: B589

Neuronal autophagosomes form via ordered assembly at transient DFCP1-positive ER subdomains in the distal axon.

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The highly polarized and complex morphology characterizing neurons poses a unique challenge to cellular trafficking pathways. Organelles and proteins must be transported across the axon with high fidelity over distances up to 1 meter. The vulnerability of the neuronal system is evidenced by the fact that mutations in axonal transport machinery lead to neuronal dysfunction and disease. While the degradative process of autophagy has been extensively examined in yeast and nonpolarized mammalian cells, less is known about the dynamics of this pathway along the axon of highly polarized neurons. We utilized live-cell imaging to investigate autophagosome biogenesis and maturation in primary dorsal root ganglion (DRG) neurons under basal conditions. Our results uncovered striking gradients of trafficking pathways along the axon, consistent with the compartmentalization of functions within the neuron. Markers for the biosynthetic pathway, such as ER exit sites, are enriched in the cell body, implicating the soma as the primary site of protein synthesis. In contrast, the formation of autophagosomes is spatially

enriched in the distal region of the axon; few autophagosomes are generated in the cell soma or mid-axon under basal conditions. Autophagosome formation proceeds via an ordered assembly of proteins recruited with stereotypical kinetics onto the developing organelle. Autophagosomes are generated from transient DFCP1-positive subdomains of the ER; DFCP1 biogenesis events are also enriched in the distal axon. Following formation, autophagosomes undergo robust transport toward the cell soma. As they move distally to proximally, autophagosomes mature into autolysosomes that more effectively catalyze cargo degradation. Delivery to the cell soma ensures efficient and rapid recycling of degraded components. This spatial and temporal regulation is not limited to actively growing DRG neurons. We also observed distal initiation followed by robust retrograde transport in synaptically-connected hippocampal neurons. Further, compartmentalization is also evident within dendrites; dendrites have few autophagosomes, which all exhibit bidirectional motility along the entire length of the process. Remarkably, autophagosome biogenesis is infrequent in dendrites suggesting that the mechanisms of autophagy in dendrites are fundamentally distinct from those in the axon. Together, our results are consistent with a highly compartmentalized pathway for constitutive autophagy along the axon of primary neurons. We propose that distal enrichment of autophagosome formation facilitates the degradation of damaged mitochondria and long-lived cytoplasmic proteins that reach the axon tip via slow axonal transport. Supported by NIH K99NS082619 to SM and NS060698 to EH.

P447

Board Number: B590

A PIK3C3/Ankyrin-B/Dynactin pathway promotes axonal growth and multi-organelle transport.

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Axon growth requires long-range transport of organelles, but how these cargos recruit their motors and how their traffic is regulated is not fully resolved. Here we identify a new pathway based on the class III PI3-kinase (PIK3C3), ankyrin-B, and dynactin that promotes fast axonal transport of synaptic vesicles, mitochondria, endosomes and lysosomes. We show that dynactin associates with cargo through ankyrin-B interactions with both the dynactin subunit p62 and phosphatidylinositol 3-phosphate lipids (PtdIns(3)P) generated by PIK3C3. Ankyrin-B knockout results in shortened axon tracts in vivo and marked reduction in membrane association of dynactin and dynein, while did not affect the organization of spectrin-actin axonal rings imaged by 3D-STORM. Loss of ankyrin-B or of its linkages to either p62 or PtdIns(3)P, or loss of PIK3C3 all impair organelle transport and particularly retrograde transport in hippocampal neurons. Our results establish new functional relationships between PIK3C3, dynactin, and ankyrin-B that together promote axonal transport of organelles and are required for normal axon length.

P448**Board Number: B591****Elevated CDK5 Activity Leads to Dysregulated Axonal Transport in Neurons.**E. Klinman¹, E.L. Holzbaur²;¹Department of Neuroscience, University of Pennsylvania School of Medicine, Philadelphia, PA,²Department of Physiology, University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA

Cargo transport is essential for neuronal function and survival. Defects in cargo transport are associated with neurodegenerative diseases including Amyotrophic Lateral Sclerosis (ALS), wherein cargo builds up in the proximal axon as a result of transport failure. Increased activity of cyclin dependent kinase 5 (CDK5) and its stress-induced activator p25 has been observed in human patients with ALS and mouse models of the disease, as well as in other neurodegenerative diseases associated with cellular stress. CDK5 interacts with both anterograde (kinesin) and retrograde (dynein) motor proteins, but the differential regulation of CDK5 in healthy and stressed cells as well as the mechanism of regulation remain unknown. Here, we show that reducing CDK5 activity in healthy dorsal root ganglia neurons affects neither directionalities nor velocities of cargo transport. A wide range of cargo was examined, including lysosomes, mitochondria, and autophagosomes, none of which responded with changes in transport when CDK5 activity was lowered. However, introducing the stress-associated CDK5 activator p25 significantly increased the number of pauses and directional switches across all examined cargo, in both the anterograde and retrograde direction. This increase in nonproductive motion is consistent with changes observed in ALS neurons, and implies a link between increased CDK5 activity in diseased cells and disruption of transport. Next, we tested whether the effects of aberrant CDK5 activation on transport were dependent on the Lis1/Ndel1 complex. This complex binds and directly regulates the activity of dynein; Ndel1 contains 5 possible CDK5 phosphorylation sites. We determined that mutating the CDK5-dependent phosphorylation sites in Ndel1 blocked the effects of p25-dependent disruption of retrograde transport for both lysosomes and mitochondria. Together, these studies identify CDK5 as a regulator of cargo transport in stressed cells, and suggest that misregulated CDK5 activity may contribute to transport disruption in neurodegenerative diseases, in an Ndel1 and Lis1-dependent fashion.

P449**Board Number: B593****Functionally distinct roles for dynactin in regulating axonal transport initiation and microtubule dynamics in the distal axon.**J.J. Nirschl¹, E.L. Holzbaur²;¹Physiology, University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA, ²Department of Physiology, University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA

The dynactin complex activates cytoplasmic dynein by promoting efficient initiation of minus-end directed vesicular transport along microtubules. Dynactin also regulates microtubule dynamics as a neuron-specific anti-catastrophe factor. Mutations in the highly conserved CAP-Gly domain of the dynactin subunit p150^{Glued} disrupt both of these functions, leading to neurodegeneration. We wondered whether the transport initiation and anti-catastrophe activities of dynactin are separable, or instead are functionally linked. To address this question, we used siRNA to deplete endogenous p150^{Glued} from primary neurons and rescued cells with constructs lacking either the N-terminal CAP-Gly domain or a flanking basic domain that also binds microtubules but with a lower affinity. Previous work has shown that the CAP-Gly domain of p150^{Glued} is required for efficient retrograde transport initiation and that the tandem microtubule-binding domains are required for dynactin-mediated effects on microtubule dynamics. However, the role of the basic domain in axonal transport has not yet been tested. Using live-cell photobleaching assays, we show that expression of a p150^{Glued} construct lacking the CAP-Gly domain rescues the retrograde transport of LAMP-1-RFP vesicles along the mid-axon but not at the distal axon, consistent with an essential role for this domain in transport initiation. In contrast, a p150^{Glued} construct lacking the basic domain effectively rescued transport in both the mid- and distal axon, indicating that this domain is not required for either transport initiation or robust vesicular transport along the axon. Thus, we can effectively separate the functions of p150^{Glued} in regulating transport initiation and microtubule dynamics: the CAP-Gly domain of p150^{Glued}, but not the basic domain, is required for retrograde transport initiation whereas both the CAP-Gly and basic domains in tandem are required for regulating dynamics at the microtubule plus-end in neurons. Thus, dynactin has two functionally separable roles in the distal axon, both of which are disrupted by human mutations that cause Perry syndrome, a lethal form of parkinsonism.

P450**Board Number: B594****A molecular mechanism for the anterograde transport of dynein in the axon.**A.E. Twelvetrees^{1,2}, G. Schiavo², E.L. Holzbaur³;¹Physiology, University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA, ²Sobell Department of Motor Neuroscience and Movement Disorders, University College London Institute of Neurology, London, United Kingdom, ³Department of Physiology, University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA

Microtubules in the axon are uniformly orientated with their plus ends directed away from the soma and towards the axon terminal. Thus, the minus end directed microtubule motor, cytoplasmic dynein, has no ability to reach the axon terminal under its own ATP hydrolysis power and must be actively transported. This process is critical to neuronal function as without the presence of dynein in axon terminals there is no facility for essential retrograde trafficking events that are key to neuronal survival. In neurones cultured from DIC1-GFP knock in mice (which express a GFP tagged form of the neuron specific dynein intermediate chain subunit, DIC1, at endogenous levels) dynein accumulates in axon terminals. By live cell TIRF-M, imaging in axons of known polarity, we observe many dynein-GFP motile events in both anterograde and retrograde directions. Previous work in the lab had identified direct interactions between kinesin-1 and the DIC subunit. Building on this observation we find multiple points of protein-protein interaction between DICs and both the light chain and heavy chain subunits of kinesin-1. These direct interactions are consistent with activation of kinesin by dynein as well as being specific to neuronal spliceforms of the DIC subunits. Consistently, we find dynein and kinesin form an endogenous complex in vesicle free brain fractions. Using FRAP analysis we find that net rates of anterograde transport for dynein populations in the axon are the same as those that typically describe slow axonal transport. We propose that neuron specific isoforms of dynein subunits support the direct activation of kinesin by dynein in order to power the anterograde transport of dynein in the axon.

P451**Board Number: B594****The Dynein Inhibitor Ciliobrevin D Inhibits the Bi-directional Transport of Organelles Along Sensory Axons and NGF Induced Axon Growth and Branching.**R. Sainath¹, G. Gallo¹;¹Shriners Hospitals Pediatric Research Center, Temple University, Philadelphia, PA

The axonal transport of organelles is critical for the development, maintenance and survival of neurons, and its dysfunction has been implicated in several neurodegenerative diseases. Retrograde axon transport is mediated by the motor protein dynein. In this study, using embryonic chicken dorsal root ganglion neurons, we investigate the effects of Ciliobrevin D, a pharmacological dynein inhibitor, on the transport of axonal organelles, axon extension, nerve growth factor (NGF)-induced branching, growth cone expansion, and axon thinning in response to actin filament depolymerization. Live imaging of

mitochondria, lysosomes and Golgi derived vesicles in axons revealed that both the retrograde and anterograde transport of these organelles was inhibited by treatment with Ciliobrevin D. Treatment with ciliobrevin D reversibly inhibited axon extension and transport, with effects detectable within the first 20 minutes of treatment. NGF induces growth cone expansion, axonal filopodia formation and branching. Ciliobrevin D prevented NGF-induced formation of axonal filopodia and branching but not growth cone expansion. Finally, we report that the retrograde reorganization of the axonal cytoplasm which occurs upon actin filament depolymerization is inhibited by treatment with Ciliobrevin D, indicating a role for microtubule based transport in this process. This study identifies Ciliobrevin D as an inhibitor of the bi-directional transport of multiple axonal organelles, indicating this drug may be a valuable tool for both the study of dynein function and a first pass analysis of the role of axonal transport.

P452

Board Number: B595

A novel role for huntingtin in the axonal transport of Rab4-containing vesicles in vivo.

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Huntingtin (HTT), the Huntington's disease (HD) protein, has been implicated in axonal transport. Previous work showed that loss of HTT function caused axonal transport defects. Biochemical and genetic experiments indicated that HTT can associate with both kinesin-1 and dynein motors either directly or via accessory proteins. However, the vesicle type or the motor-cargo complex that HTT is contained in during axonal transport is unknown. Using *in vivo* motility analysis in *Drosophila* larval axons, we found that HTT influences the movement of particular Rab containing-vesicles. Interestingly, while reduction of HTT dramatically perturbed the bi-directional movement of Rab3 and Rab4 containing vesicles, only the retrograde movement of Rab7 and Rab19 containing vesicles were perturbed. Rab19 is proposed to be on recycling endosomes, while Rab3 is thought to be on synaptic vesicles. The identity of Rab4-containing vesicles is unknown. Using dual-color imaging we examined whether HTT is physically present on moving Rab-containing vesicles. While Rab4 is present on most of the HTT containing vesicles, not all Rab4 vesicles contained HTT. Further Rab4 is also present on synaptotagmine and synaptobrevin containing vesicles indicating that Rab4 is likely to be on synaptic vesicles. Both kinesin and dynein motors are also required for the movement of Rab4 containing vesicles. Collectively, our *in vivo* analysis provides compelling evidence for a moving HTT-Rab4 motor-complex during axonal transport and suggests that HTT may likely influence the motility of different Rab-containing vesicles. Moreover, expansion of polyQ repeats in the context of HTT dramatically perturbed the motility of Rab4-containing vesicles indicating that disruption of Rab transport mediated by mutant HTT could contribute to the neuropathology observed in HD.

P453**Board Number: B596****Directional actin turnover transports actin and associated proteins for early axonal outgrowth.**H. Katsuno¹, M. Toriyama², Y. Hosokawa³, K. Mizuno⁴, K. Ikeda⁵, Y. Sakumura^{6,7}, N. Inagaki⁷;¹Grad. Sch. Biol. Sci., Nara Institute of Science and Technology, Ikoma, Japan, ²Molecular Biosciences, University of Texas at Austin, Austin, TX, ³Grad. Sch. Mater. Sci., Nara Institute of Science and Technology, Ikoma, Japan, ⁴Tohoku Univ., Sendai, Japan, ⁵Grad. Sch. Inform.Sci., Nara Institute of Science and Technology, Ikoma, Japan, ⁶Sch. Inform. Sci. and Technol., Aichi Prefectural Univ., Nagakute, Japan, ⁷Grad. Sch. Bio. Sci., Nara Institute of Science and Technology, Ikoma, Japan

Intracellular transport is fundamental to cellular activities, supplying essential components to functionally specialized regions. Motor proteins, such as myosin and kinesin, walk along cytoskeletons to achieve directional transport of membrane organelles and protein complexes. Although actin and associated proteins at cellular leading edge are essential for cell protrusion and motility, how they are transported to the subcellular regions remain unclear. Previous report showed that an actin rich structure called “wave” moves along the axonal shafts or actin and associated proteins (Ruthel & Banker, *Cell Motil. Cytoskeleton*, 1998; Toriyama et al., *J. Cell Biol.*, 2006; Flyn et al., *Dev. Neurobiol.*, 2009)

Here we show that assemblies of F-actins migrating along axonal shafts are transported toward the growth cone of extending axons by means of their directional polymerization/depolymerization (treadmilling), a mechanism distinct from the motor-based ones. We found that the anterogradely migrating F-actins undergo treadmilling, with their polymerizing ends pointing toward the neurite tips. The lateral sides of F-actins were mechanically anchored to the plasma membrane and substrates through a linker protein shootin1 and a cell adhesion molecule L1-CAM. The velocity of actin transport positively correlated with the rate of actin polymerization and the degree of F-actin anchoring to the substrate. We also detected directional counter-force associated with the F-actin transport on the substrate through their anchorage. Actin interacting protein cofilin, cortactin and shootin1 co-migrated with F-actins in an actin-transport-dependent manner. Furthermore, blocking this transport by creating an adhesion gap along the axonal shaft inhibited axonal elongation. These findings present a novel mechanism for intracellular transport, which supplies actin and associated proteins toward cellular leading edge, thereby promoting its protrusive activity.

P454**Board Number: B597****Glucose Regulates Mitochondrial Motility via Milton Modification by O-GlcNAc Transferase.**G. Pekkurnaz¹, J.C. Trinidad², X. Wang³, D. Kong⁴, T.L. Schwarz^{1,5};¹The F.M. Kirby Neurobiology Center, Boston Children's Hospital, Boston, MA, ²Department of Chemistry, Indiana University, Bloomington, IN, ³Department of Neurosurgery, Stanford University, Palo Alto, CA, ⁴Department of Neuroscience, Tufts University School of Medicine, Boston, MA, ⁵Department of Neurobiology, Harvard Medical School, Boston, MA

Cellular homeostasis critically depends on the ability of cells to monitor nutrient levels that can be used for ATP generation by mitochondria. Among the many specialized cell types, neurons are particularly dependent on mitochondria due to their complex morphology and regional energy needs. Hence, abnormalities in mitochondrial distribution have been implicated in many neuropathologies including Alzheimer's, Parkinson's, and Huntington's diseases. Here, we report a molecular mechanism by which nutrient availability in the form of extracellular glucose regulates mitochondrial motility in neurons. We show that this mechanism requires the activity of O-GlcNAc Transferase (OGT), an enzyme whose activity depends on glucose availability, and that activation of OGT diminishes mitochondrial motility. We establish the mitochondrial motor adaptor protein Milton as a required substrate for OGT to arrest mitochondrial motility by mapping and mutating the key O-GlcNAcylated serine residues. We find that the O-GlcNAcylation state of Milton is altered by extracellular glucose, pharmacological inhibition of O-GlcNAcase or UDP-GlcNAc synthesis, and that OGT alters mitochondrial motility *in vivo*. Our findings suggest that, by dynamically regulating Milton O-GlcNAcylation, OGT tailors mitochondrial dynamics in neurons based on nutrient availability. Although in this study we took advantage of the polarized and regular arrays of microtubules in axons, the components of this pathway are present in all metazoan cells, and glucose, via OGT, may therefore be a widespread regulator of mitochondrial movement.

P455**Board Number: B600****Bidirectional cargo transport: Moving beyond tug-of-war.**W.O. Hancock^{1,2};¹Department of Biomedical Engineering, Pennsylvania State University, University Park, PA,²Interdisciplinary Graduate Degree Program in Bioengineering, Pennsylvania State University, University Park, PA

Bidirectional transport of vesicles, organelles and other intracellular cargo along microtubules is frequently described as a "tug-of-war" between oppositely-directed kinesin and dynein motors attached to the same cargo. A number of experimental and modeling studies support the tug-of-war paradigm, and it is the dominant model in the field. However, numerous knockout and inhibition studies in a variety of systems have found that inhibiting one motor leads to diminished motility in both directions.

This phenomenon can be defined as a “paradox of codependence” that directly challenges the tug of war paradigm. To resolve it, alternative models are needed that move beyond the tug of war paradigm. In an effort to resolve the paradox of co-dependence, I will describe three classes of bidirectional transport models, termed microtubule tethering, mechanical activation, and steric disinhibition (1). For each mechanism, experimental support from the literature will be presented, along with approaches for developing computational models that can be used to generate experimental predictions and interpret data. These proposed mechanisms and the development of models based on them will help to guide future experiments aimed at uncovering the fundamental mechanisms underlying bidirectional transport. (1) W.O. Hancock, Nature Reviews Molecular Cell Biology, September, 2014.

P456

Board Number: B601

Is the axonal transport of neurofilaments a tug-of-war between opposing motors?

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Neurofilaments are space-filling cytoskeletal polymers in nerve cells that function to increase axon caliber, thereby maximizing the rate of propagation of the nerve impulse. In addition of their structural role, these polymers are also transported along axons in a stop-and-go manner, exhibiting brief bouts of rapid bidirectional movement interrupted by prolonged pauses. The filaments move on microtubule tracks powered by kinesin-1 in the anterograde direction and dynein in the retrograde direction. To investigate the motile mechanism, we transfected primary cultures of cortical neurons from neonatal rat brains with GFP-tagged neurofilament protein M and then recorded neurofilament movement by epifluorescence microscopy at 30 frames per second using an EMCCD camera. We used kymograph analysis to track neurofilament movement, and edge detection algorithms to extract the paths of the leading and trailing ends of the moving filaments. We developed an unbiased noise-filtering algorithm to extract bouts of movement and pauses, and then used this method to obtain statistical distributions of pause times, run times and velocities. Remarkably, we found that the overall average velocities, the average velocities during a contiguous run, the run length distributions and the pause time distributions were all independent of neurofilament length across a range of lengths from 1-36 μm . To investigate whether the observed motile behavior can be generated by a tug-of-war, we developed a computational model in which dynein and kinesin motors attach to a single neurofilament cargo and interact through mechanical forces only. We then systematically varied the number of kinesin and dynein motors in the model and attempted to identify those combinations of motors that matched the neurofilament velocity, pause time and run length distributions in each kymograph trace. So far we have found no inconsistencies between the tug-of-war model and the experimental data, but distinct kinetic phases within individual traces suggest that the number of motors bound to the neurofilament cargo is not fixed and may change over time. The model predicts that the total number of active motors on each

neurofilament is relatively small and relatively independent of polymer length. Thus the motors may not be distributed uniformly along the filaments.

P457

Board Number: B602

Witnessing microtubule-based transport in the living brain: Impact of the cargo-motor receptor, amyloid precursor protein, and Alzheimer's plaques.

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Most amyloid precursor protein (APP)-based Alzheimer's models overexpress mutant human APP resulting in Aβ plaques. Yet the relative contribution of this elevated APP and the presence of plaques to neurodegeneration remains a big question. APP's role as a cargo-motor receptor for axonal transport suggests that overexpression might lead to increased transport. Indeed we showed that transport is increased in Down's syndrome and decreased in APP knockout mice. Hence transport may be elevated in APP overexpressors and lead to either beneficial or deleterious consequences. Here we use high field microMRI with Mn²⁺, an MR contrast agent useful as a track-tracer, to pose this cell biological question within the whole living brains of wildtype and Alzheimer's model mice. Injection of Mn²⁺ into the CA3 region of the hippocampus results in measurable transport over time. Application of 3D unbiased whole brain image analysis detects all circuitry emanating from the hippocampus. By driving APP Swe/Ind transgene expression with a tetracycline-sensitive promoter, APPSwe/Ind expression can be decoupled from the presence of plaques with doxycycline (doxy). Three groups of mice were studied: group 'A' (no doxy, +plaques, +APP); group 'B' (doxy at 8 days before sacrifice, +plaques, no APP), and group 'C' (doxy prior to conception, and stopped 8 days before sacrifice, no plaques, +APP). Images were captured before and sequentially after Mn²⁺ injection into CA3 (1, 7, 25 hr). Images were aligned and analyzed by statistical parametric mapping to identify differential accumulation within the hippocampal projections. Histopathology revealed well-developed plaques in A and B, and Western blots showed human APP expressed five-fold over WT in in A and C. Our preliminary results show increased transport in A and C, with APP Swe/Ind expression when compared with B, where expression is suppressed. Cholinergic neurons in the medial septal nucleus were decreased as determined by anti-ChAT staining in Group C (p=0.0006 by one-way ANOVA, n=15). In conclusion, the effects of elevated APP expression are separable from consequences of plaque, and each may. Supported by NIGMS P50GM08273 and NINDS NS062184.

P458**Board Number: B603****Mobility of axonal neurofilaments in mature adult myelinated axons.**J. Fenn¹, P. Jung², J. Julien³, A. Brown¹;¹Department of Neuroscience, The Ohio State University, Columbus, OH, ²Quantitative Biology Institute, Ohio University, Athens, OH, ³Department of Psychiatry and Neuroscience, Laval University, Quebec, QC

Neurofilaments are the intermediate filaments of neurons and serve as critical structural elements that support the growth of axon caliber, which is an important determinant of axonal conduction velocity. Abnormal accumulations of neurofilaments are frequently manifested in neurological disorders such as amyotrophic lateral sclerosis, giant axonal neuropathy, diabetic neuropathy, Charcot-Marie-Tooth disease, Alzheimer's disease and others. For many years neurofilaments were thought to behave in a relatively static manner in mature neurons. Early electron microscopic evidence even suggested that neurofilaments may be immobile because they appeared to form a mesh-like network. We now know that neurofilaments are transported along axons, but there is still debate regarding what proportion of these polymers move. Some researchers have argued that most axonally transported neurofilaments are deposited into a permanently stationary network with less than 10% capable of movement. In contrast, we have argued that there are no permanently stationary axonal neurofilaments and that essentially all of these polymers are intermittently mobile, cycling repeatedly between moving and pausing states throughout their lifetime. Live cell imaging studies on cultured neurons from embryonic and neonatal animals support this more dynamic perspective, but it has been argued that this may not hold true for mature myelinated axons in vivo. To resolve this controversy, we have used a pulse-escape fluorescence photoactivation technique to analyze neurofilament transport in intact peripheral nerves of adult Thy1-paGFP-NFM mice, which express neurofilament protein M tagged with a photoactivatable GFP. Neurofilaments were activated in 5 μ m-long segments of myelinated axons (average diameter= 3.3 μ m) and the mobility of the polymers was determined by analyzing the fluorescence remaining in the activated window over time. Preliminary data show that 83 \pm 12% of the neurofilaments (n=21 axons) departed from the window within 3 hours after activation, indicating a highly mobile population. Thus, any permanently stationary neurofilaments cannot exceed 17% of the total population in these axons. Moreover, by extrapolation of the decay kinetics, we predict that all of the neurofilaments would have departed the activation window within 12 hours. We therefore conclude that most, if not all, axonal neurofilaments are mobile, even in mature myelinated axons of adult animals. We believe that this dynamic cytoskeletal architecture has important implications for the mechanism by which axon morphology is regulated during development and maturation, and by which neurofilaments accumulate in disease states.

P459**Board Number: B604****SMN functions as a chaperone for mRNP complex assembly.**P.G. Donlin-Asp¹, C. Fallini^{1,2}, J.P. Rouanet¹, K.R. Williams¹, G.J. Bassell^{1,3}, W. Rossoll^{1,3};¹Cell Biology, Emory University School of Medicine, Atlanta, GA, ²Neurology, University of Massachusetts Medical School, Worcester, MA, ³Neurology, Emory University School of Medicine, Atlanta, GA

Spinal muscular atrophy (SMA) is a neuromuscular disease characterized by a specific degeneration of motor neurons. SMA results from a reduction in the survival of motor neuron (SMN) protein, which is ubiquitously expressed with a well characterized role in promoting the assembly of spliceosomal small nuclear ribonucleoproteins (snRNPs). While underlying defects in splicing have been observed in SMA models, these defects are not unique to motor neurons, leaving their role in the selective motor neuron degeneration unclear. Studies from our laboratory and others have revealed impairments in axonal mRNA localization in SMA models. We have discovered specific defects in the axonal localization of mRNAs (*β-actin*, *Gap43*) and mRNA-binding proteins (HuD, IMP1) in axons and growth cones of primary motor neurons from SMA mice or depleted of SMN. We also observed that overexpression of both HuD and IMP1 can mitigate the effects of low SMN levels on axon outgrowth and GAP43 protein levels in growth cones. These findings led to the hypothesis that SMN plays a critical role in the assembly and/or trafficking of messenger ribonucleoproteins (mRNPs) in neuronal processes. However, the molecular function(s) of SMN in the biology of mRNPs remained unclear due to the lack of specific assays. To investigate the biological role of SMN and the effects of SMN deficiency in axonal mRNA regulation, we have established a trimolecular fluorescence complementation (TriFC) assay in motor neuron cultures as a sensor for mRNA and protein association, which permitted an analysis of the proposed role of SMN in mRNP assembly and localization. Our findings revealed a deficiency in the assembly of IMP1 protein / *β-actin* mRNA containing complexes in SMA motor neurons, which is consistent with SMN acting as a chaperone for mRNP complex assembly. These results are further supported by RNA immunoprecipitation experiments from SMA mouse tissue extracts, which show a substantial reduction in the levels of *β-actin* and *Gap43* mRNAs associated with IMP1. We further show that SMN-deficiency results in defects in axonal local translation, likely a downstream consequence of impaired mRNP transport complex assembly. Local translation of *β-actin* and *GAP43* are known to play an important role in axon outgrowth and branching. Our findings reveal a novel function for SMN in the assembly of axonally transported mRNP complexes, and uncover consequent effects on axonal mRNA localization and translation that may contribute to SMA pathology.

Receptors, Transporters, and Channels

P460

Board Number: B606

Determining the key residues within the MUC1 transmembrane domain dimer.

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Overexpression of the membrane protein mucin 1 (MUC1) has been linked to 75% of all human solid tumor cancers, including 90% of breast carcinomas. In cancer cells, MUC1 homodimerization has been associated with cell migration and adhesion. Furthermore, this interaction is necessary to form complexes with growth factor receptors targeting the nucleus, where MUC1 can interact with effector proteins regulating gene expression. Thus, understanding how MUC1 forms dimers is essential for developing novel therapeutic strategies to inhibit its oncogenic effects. Therefore, we are using site-directed mutagenesis and the ToxR assay to study certain residues within the transmembrane domain of MUC1 and their contribution to dimerization.

P461

Board Number: B607

Proteomic and Functional Analyses of the Acidocalcisome, an Organelle Conserved from Bacterial to Human Cells.

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Acidocalcisomes are acidic organelles present in a diverse range of organisms from bacterial to human cells. In this study acidocalcisomes were purified from the model organism *Trypanosoma brucei*, and their protein composition was determined by mass spectrometry. The results, along with those that we previously reported, show that acidocalcisomes are rich in pumps and transporters, involved in phosphate and cation homeostasis, and calcium signaling. We validated the acidocalcisome localization of several proteins by expressing them fused to epitope tags in their endogenous loci or by immunofluorescence microscopy with specific antibodies. Knockdown of several newly identified acidocalcisome proteins by RNA interference (RNAi) revealed that they are essential for the survival of the parasites. These results provide the first comprehensive insight into the unique composition of acidocalcisomes of *T. brucei*, an important eukaryotic pathogen.

P462**Board Number: B608****Y1: a potential target of endocannabinoids.**A. Naeem¹, M.J. Khan¹, A. Khan¹;¹Department of Biosciences, COMSATS Institute of Information Technology Islamabad, Islamabad, Pakistan

NPY is 36 amino acid proteins having six receptors residing in hypothalamus. Y1 (Neuropeptide Y receptor 1) is a G-coupled transmembrane receptor that controls the regulation of feed intake thus playing a crucial role in obesity. Y1 is regulated by one of the neuropeptide protein NPY that is its natural ligand. Many drugs like BIBP3226, SR141716 and BIBO3304 have been introduced in the market as an inhibitor of Y1 to control feed intake. Another well-known system for appetite regulation is endocannabinoid system (ECS) that is regulated by endocannabinoids (ECs). The ECs are natural ligands of cannabinoid receptor 1 (CB1) located in hypothalamus. However, EC are reported to bind to Y1 and may potentially inhibit Y1 signaling. In this study, we have explored the interaction of thirty-five endocannabinoids with Y1 and CB1. For all the selected ECs, blood brain barrier (BBB) permeability was evaluated through their chemical properties. Docking analysis of the EC ligands showed that all ECs exhibit strong binding with CB1. Remarkably, ECs displayed high affinity towards Y1 as well and displayed binding energies that were comparable to CB1. On the basis of binding energy, interactions and ability to cross BBB, gamma-linolenoyl ethanolamide (BE -6.6 with Y1 and -4.4 with CB1), oleoyl-ethanolamide-d4 (BE of -5.3 with Y1 and -4.2 with CB1) and oleamide (BE of -5.6 with Y1 and -4 with CB1) were identified as most active ECs. Thus, endocannabinoids regulate appetite stimulation through CB1 signaling, potentially play the opposite role through Y1 signaling and provide the delicate balance between the two main pathways; that governs the energy intake regulation.

Keywords: NPY, Y1, Neuropeptide Y1, CB1, Endocannabinoids.

P463**Board Number: B609****B lymphocytes from lupus-prone mice are highly sensitive to LPS stimulation.**P. Eisenhauer¹, A. Kane¹, R.A. Roberts²;¹Ursinus College, Collegeville, PA, ²Biology, Ursinus College, Collegeville, PA

Systemic Lupus Erythematosus (SLE) is an autoimmune disease characterized by immune hyperactivity that produces a wide range of symptoms. Bacterial lipopolysaccharides (LPS) have been shown to initiate various immune responses and may play a role in SLE pathogenesis. This research sought to determine if LPS treatment has an effect on populations of splenic B lymphocytes, as well as an effect on the levels of MHC-II and B220 surface molecule expression, in control (C57BL/6) and lupus-prone (NZB/WF1) mice. Splenic B-lymphocytes were treated with 10 µg/mL or 20 µg/mL LPS for 18 hours in charcoal-treated phenol red-free fetal calf serum. Fluorescent Activated Cell Scanning (FACS) was

performed to measure B lymphocyte populations and surface protein expression. Compared to control, lupus-prone mice express a lower proportion of B lymphocytes per total cell population, as measured by B220 expression and confirmed by CD19 surface molecule expression. Further, a lower percentage of B lymphocytes express MHC-II in lupus-prone cell populations compared to control cell populations. However, lupus-prone cell populations appear to be more sensitive to LPS stimulation, showing increased blasting and increased MHC-II expression compared to control cells following LPS exposure. These results suggest that B lymphocytes in NZB/WF1 mice, while smaller in total cell proportion compared to control, may be more reactive to environmental stimulants such as LPS.

P464

Board Number: B610

Is AMH signaling modulated by TGFB superfamily binding proteins?.

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Anti-Müllerian hormone (AMH) is a gonadal hormone that induces part of the male phenotype. However, it is also present in the blood of both men and women, and is a putative local regulator of adult ovarian and testicular function. This suggests that AMH, like other members of the Transforming growth factor beta (TGFB) superfamily, is a pleiotropic regulator. The TGFB superfamily ligands share receptors and binding proteins (BPs), leading to context-dependent signaling. AMH is the only ligand to have a unique type 2 receptor (AMHR2), but it shares type 1 receptors with other TGFBs. Its ability to interact with other TGFB superfamily members through common BPs is unknown. AMH has a precursor form (proAMH) in blood, and also exists as a mature complex (AMH_{N,C}) that dissociates when AMH_C binds to AMHR2. We have therefore examined the influence of TGFB BPs on the various forms of AMH. Fourteen TGFB BPs were examined in two assays. The influence of BPs on AMH_C or AMH_{N,C} receptor binding was measured using a bone morphogenetic protein response element (BRE) luciferase reporter assay. Western blots were used to measure proAMH cleavage to AMH_{N,C} in the presence of BPs. Several of the BPs had subtle effects on receptor activation, with the follistatins increasing and endoglin decreasing EC₅₀, by 25-35%. These BPs were effective at AMH concentrations present in adult blood (50pM) but not at boy levels (1nM). The densities of the AMH_{N,C} westernbands generated by furin cleavage of proAMH were not altered by the addition of any of the fourteen BPs, suggesting that the activation of proAMH is not modulated by known TGFB BPs. In conclusion, AMH may share some of the BPs that modulate and integrate the activities of the TGFB superfamily. The physiological actions of the BPs may be limited to adults.

P465**Board Number: B611****HUMAN PLATELET ESTROGEN RECEPTORS: LOCALIZATION AND THEIR ROLE IN AGGREGATION.**N.H. Patel¹, R.A. Mitteer², J.M. Prakash², O. Borodevyc², G. Soslau³, H. Singh⁴;¹Department of Internal Medicine, Drexel University College of Medicine, Philadelphia, PA, ²Office of Professional Studies in the Health Sciences, Drexel University College of Medicine, Philadelphia, PA,³Office of Professional Studies in the Health Sciences/Biochemistry and Molecular Biology, Drexel University College of Medicine, Philadelphia, PA, ⁴Pharmacology and Physiology, Drexel University College of Medicine, Philadelphia, PA

Platelets are vital to thrombosis and the pathophysiology of coronary artery disease (CAD). Platelet membranes are dynamic structures involved in regulating aggregation and thrombosis. Agonists bind to surface receptors, activating second messenger systems that result in platelet aggregation.

Gender differences in CAD have been attributed to the effects of estrogen. Paradoxically, some in vitro studies report that estrogen upregulates platelet activity while others report the opposite. Using aggregometry assays, we found that exposure to physiologic levels (60-100nM) of 17- β -estradiol (E2) initially increased agonist induced activation; with prolonged E2 exposure this effect is reversed. E2 exposure alone did not induce any aggregation. Additionally, lipid rafts are necessary for this initial synergy to occur. Classically, steroids like E2 regulate nuclear gene transcription. Platelets are anucleate – thus there must exist a non-genomic mechanism of steroids. We tested the hypothesis that platelets possess membrane bound estrogen receptors (ERs) capable of modulating platelet activity.

Platelet rich plasma (PRP) was isolated by centrifugation of whole blood from donors at 400g. PRP was then diluted and fixed for immunofluorescent microscopy using 4% (w/v) paraformaldehyde. Using antibodies to 3 major ERs – ER α , ER β , and G Protein Coupled Receptor 30 (GPR30) – we showed that platelet membranes possess all 3 ERs by immunocytochemistry and western blots. Furthermore, GPR30 is present on the cytosolic membrane face, while ER α and ER β are exposed on the external surface.

PRP from males was incubated with E2 and slides again prepped with a focus on the colocalization of ERs to Glycoprotein IIb/IIIa (GPIIb/IIIa). GPIIb/IIIa is the most abundant protein found exclusively on the platelet membrane, and plays a crucial role in aggregation. On short (3 minutes) exposure to E2, ER colocalization with GPIIb/IIIa increases. This was most prominent with GPR30.

Platelets were then activated with Thrombin Receptor Activating Peptide-1 (TRAP1), a synthetic thrombin analog that acts via Protease Activated Receptor-1 (PAR1) to activate GPIIb/IIIa. TRAP1 activation in E2 treated platelets resulted in less colocalization of ERs with GPIIb/IIIa compared to resting E2 treated platelets.

Our results suggest that E2 upregulates aggregation by priming the GPIIb/IIIa complex. Lipid rafts may mediate migration of ERs towards GPIIb/IIIa. Upon activation, uncoupling of ERs from GPIIb/IIIa begins and may explain the time dependent decrease in aggregation, translating to a cardioprotective effect of

estrogen. In this study we have established that ER α , ER β and GPR30 localized in platelets play a direct role in platelet aggregation and can be used as therapeutic targets in CAD.

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Board Number: B612

Ion channel formation by mouse prion protein; Effects on membrane permeability and cell toxicity.

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Prion diseases are a group of fatal neurodegenerative disorders which affect animals and humans. It is well accepted that the conversion of native monomeric cellular prion protein (PrPC) to an aggregated oligomeric form (PrPSc) elicits the pathological features in these diseases. It has been proposed that direct interaction between PrP and lipid membrane, apart from the GPI-anchor, might play a critical role in the PrPSc formation. Peptides derived from the PrP have been shown to interact with lipid membrane and many of these peptides elicit cell toxicity by perturbing the ionic balance inside the cell. In this study, we have shown that GPI- anchor less full length mouse prion protein (moPrP) bind to lipid membrane and causes calcium release from the lipid vesicles. Also, we have shown that, using a BLM technique, moPrP forms a cation selective channel which allows the transit of only potassium and calcium ions. Surprisingly, we have observed that the exogenous addition of moPrP into HEK 293T cells leads to the incorporation of moPrP into the membrane and forms ion channels. However, we have also observed an immediate increase in the intracellular calcium level. Importantly, this dynamic increase of the intracellular calcium level is followed a pattern which is similar to the typical calcium signaling. This observation suggests that prion protein is not only able to form ion channels on the membrane but could also activate calcium signaling through a group of receptor/s. Perhaps, these combinations of both the ion channel formation and signaling activity of moPrP might be sufficient to trigger the apoptotic pathways through the discharge of cellular membrane potential, specifically, mediated by the calcium influx. We have also shown that moPrP aggregates interact with the membrane and forms pores which can finally rupture the membrane.

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Board Number: B613

Inhibition of ChLoride Intracellular Channel (CLIC) Proteins by IAA-94 Induce Reactive Oxygen Species Release from Cardiac Mitochondria.

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Reactive oxygen species (ROS) are known to play a key role in modulating cell signaling pathways and, when produced at high levels, they induce the development of disease states such as atherosclerosis,

ischemic injury, cardiac hypertrophy and hypertension and cell death. In mammalian cells, mitochondria are a major source of ROS. A small amount of leaked electrons from the generally efficient electron transport chain (ETC) participate in reduction of oxygen to superoxide mostly in complex III and, due to the back flow of electrons, in complex I. In cardioprotection, modulation of ROS production is controlled by activation of mitochondrial ion channels. Even though ChLoride Intracellular Ion Channel (CLIC) proteins are predicted to localize to the mitochondria, there is no evidence of involvement of CLICs in ROS modulation in cardiac mitochondria. In this study, we focus on the role of CLICs in mitochondrial ROS production. CLIC proteins are present in mammals (CLIC1-6) and are regulated by redox potential. R(+)-Indanyloxyacetic acid 94 (IAA-94), a known CLIC blocker is shown to play an inhibitory role in cardioprotection. Here, using specific antibodies, we demonstrate the expression and localization of CLICs in mitochondria of neonatal and adult cardiomyocytes isolated from rats and in cardiac tubes of *Drosophila* by Western blots and immunocytochemistry. To establish the role of CLICs in mitochondrial ROS production, we measured ROS production from isolated cardiac mitochondria using amplex red dye (Invitrogen). We found that, in the presence of the complex II/III substrate (3 mM, succinate) and complex I substrate (5mM, glutamate/malate), addition of 100 μ M IAA-94 resulted in a robust release of ROS from mitochondria. The EC₅₀ for IAA-94 was found to be 16.5 \pm 0.15 μ M (n=3) for ROS produced in presence of succinate. After an initial fast release (ROS spike) there was an 82.5% (n=3) reduction in rate of ROS production with succinate as a substrate; however there was an 18.1% increase (n=3) in rate of ROS production with glutamate- malate as a substrate for 100 μ M IAA-94. We also reconstituted mitochondrial membrane proteins in a planar lipid bilayer, and discovered that IAA-94 partially blocked channel activity that could arise from CLIC-like proteins. Furthermore, we found that CLIC5 is localized to the mitochondria of neonatal and adult cardiomyocytes. The molecular identity of an inner mitochondrial anion channel (iMAC) has not yet been established, and our results indicate that CLIC5 could be a putative candidate for an iMAC. The differential effects of mitochondrial ROS production by blocking CLICs, and its impact on cardiac mitochondrial physiology may help to determine specific therapeutic strategies for cardioprotection.

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Board Number: B614

Manipulation of the luminal domain of IRE1 changes the quality of UPR signaling.

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In response to endoplasmic reticulum (ER) stress, the unfolded protein response (UPR) signals to re-adjusts the protein folding capacity of the ER. If the re-adjustment fails, the cells are committed to apoptosis. One of the major UPR signal transducers, the transmembrane kinase-endoribonuclease inositol-requiring enzyme 1 (IRE1), dimerizes and oligomerizes in response to ER stress, and depending on the magnitude of the stress IRE1 activates either pro-survival or pro-apoptotic signaling. However, it

is still unknown how IRE1 shifts from the dimer state to the oligomer, and whether the dimer is responsible for pro-survival UPR signaling and the oligomer for the apoptotic outcome. Here, we investigated how changes in the luminal domain of IRE1, which senses ER stress, underlie this molecular and/or functional transition. The substitutions D123P and K121Y, previously shown to inhibit signaling by IRE1, abrogate the clustering of IRE1 that is normally induced by chemical ER stress, but do not abolish XBP1 splicing. This suggests that splicing can be performed by oligomeric species of IRE1 that are not detectable by microscopy (perhaps dimers). The D123P mutant is also defective in the RIDD activity of IRE1 (the degradation of ER-bound transcripts). On the other hand, another mutation in the luminal domain of IRE1, C148S, which we previously showed prolongs XBP1 splicing, does not affect the capacity of IRE1 to form clusters. Therefore, D123P and K121Y display a selective phenotype. Expression of a N-terminal luminal domain (NLD) of IRE1 also inhibits IRE1 clustering, but in addition inhibits XBP1 splicing. The inhibitory effect of NLD expression relies on a physical interaction with full-length IRE1 and is observed already at a 1:1 expression ratio. A truncated NLD that does not contain the core stress-sensing region is still able to inhibit IRE1 and the inhibition is specific to IRE1, because expression of NLD does not impact the activity of the related UPR sensor, the kinase PERK. Taken together, these data suggest that: (1) Manipulation of the luminal domain affects the output of IRE1, which is mediated by the cytosolic domains. (2) XBP1 Splicing and RIDD activity can be differentiated and D123P IRE1 is able to perform the former but not the latter, perhaps because it dimerizes but does not oligomerize. We hypothesize that the precise manner by which IRE1 luminal domains interact upon ER stress determines whether the outcome of signaling is adaptive or pro-apoptotic.

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P469

Board Number: B615

Regulation of epithelial ion channels by the ER luminal chaperone ERp29.

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ERp29 is an endoplasmic reticulum (ER) 29 kD thioredoxin-homologous protein. Interestingly, ERp29 has only a single Cysteine instead of the usual C-X-X-C thioredoxins motif. ERp29 displays chaperone-like properties at both the biophysical and cellular levels and plays a role in regulating connexin 43 hemichannel biogenesis and assembly. Our group has also demonstrated that ERp29 promotes CFTR biogenesis. As the biogenesis of CFTR and ENaC share similar features, the present studies tested the hypothesis that ERp29 would also regulate ENaC biogenesis and functional expression, as well as further probed the mechanism by which ERp29 promotes both ENaC and CFTR biogenesis. In MDCK epithelial cells expressing $\alpha\beta\gamma$ -ENaC, overexpression of wt ERp29 increased the abundance of the active form of γ -ENaC, as well as ENaC functional expression in Ussing chamber experiments. In contrast, ERp29 overexpression of a mutant ERp29 lacking its single Cysteine (C157S ERp29) decreased ENaC functional expression. These observations were not associated with altered expression of β -ENaC at the apical

surface, suggesting that ERp29 may modulate ENaC open probability at the apical surface. Instead, ERp29 overexpression promoted the interaction of both ENaC and CFTR with the coat complex II ER exit machinery, whereas C157S ERp29 overexpression decreased this interaction. Finally, we also tested the hypothesis that ERp29's escape from ER retention is critical for its regulation of CFTR. We therefore designed a mutant containing a KDEL retention motif (ERp29 KDEL) and two mutants that would better escape the ER, by mutation and deletion (ERp29 KDEV and DKEEL, respectively). Together our findings suggest a key role for ERp29 in the biogenesis of ENaC and CFTR as well as emphasize ERp29 mechanism.

P470

Board Number: B616

Exosomes as delivery vesicles for transferrin and lactoferrin into mammalian cells.

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Exosomes are vesicles of endocytic origin formed by invagination of cell membrane into endosomes to form multivesicular bodies (MVBs). These small vesicles (30-120nm) are released from the cells when MVBs fuse with cell surface. Exosomes are secreted by all types of cells in culture, and are also found in abundance in body fluids including blood, saliva, urine, and breast milk. They are concentrated carriers of genetic and proteomic information, and thus are believed to play important roles in intercellular communication. Exosomes are involved in numerous functions such as signaling, activation of the target cells, delivery of information via miRNA and delivery of proteins to different cells of body. Currently exosomes are being extensively studied for delivery of different molecules (siRNA and drugs). Iron is an indispensable element required by all organisms for vital metabolic processes. The properties that make iron essential also make it toxic as free iron has the ability to produce oxidative radicals. Almost all the iron present in body fluid is bound to either transferrin (Tf) or lactoferrin (Lf) and the controlled uptake of these proteins via cell surface receptors contributes significantly in maintaining iron homeostasis. The present study attempts to characterize the role of exosome's in Tf and Lf delivery. For this, exosomes from the culture supernatant of various cell types were purified and we found that not only were exosomes capable of capturing Tf & Lf but could also deliver these iron carrier proteins more efficiently into different mammalian cells. Enhanced uptake of Tf & Lf by internalization of exosomes into cells led to increased iron acquisition by cells. Uptake of exosome associated Tf and Lf was found to be both dose and time dependent. Previously, we have reported that the multifunctional Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) recruited onto mammalian cell surface as well as in a secreted form functions as a novel receptor for Tf & Lf leading to trafficking of these proteins into cells. Proteomic profiling of exosomes from different origins has demonstrated the presence of GAPDH. Here we also confirmed the presence of GAPDH in exosomes and demonstrated that exosomal GAPDH is involved in

interaction with Tf & Lf. Interaction of exosomal GAPDH with Tf & Lf was confirmed by co-immunoprecipitation and GAPDH supplemented exosomes had a higher loading of these proteins compared to control exosomes. This is the first report demonstrating, that exosomes functions as nano vesicle for the delivery of Tf & Lf into mammalian cells. Understanding of this pathway may provide a new insight into human disorders associated with iron deficiency.

P471

Board Number: B617

Investigating the regulation of Notch activation at the cell surface: Single molecule tracking and ensemble imaging reveal membrane dynamics and spatial organization of the Notch receptor.

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Notch signaling is a highly conserved cell-to-cell communication mechanism throughout the metazoan. Notch plays a fundamental role in regulating cell response to developmental cues, apoptotic signals, and growth stimuli. Signals exchanged between neighboring cells via Notch amplify cellular differences, tipping the scales of cell fate determination and facilitating short-range pattern formation in development. Aberrant Notch signaling is linked to several developmental and adult onset disorders, as well as cancer.

Unlike many cell surface receptors, Notch, a type I transmembrane receptor, is activated by ligands presented as transmembrane proteins on apposing cells, rather than by soluble ligands. This unique aspect of Notch activation restricts signaling to cells that physically contact each other and is an example of Juxtacrine signaling. Because of the requirement for contact, spatial organization of the Notch ligands and receptors the cell has the capability to regulate activation by controlling the spatial arrangement of Notch and activation pathway components, including ligand and activating proteases. While the biochemical interactions of Notch activation pathway are fairly well understood, the mechanisms involved in spatial regulation and dynamic interactions of Notch are less well characterized despite many proposed models. As such, we are investigating the spatial organization and dynamics of Notch at the cell surface using both single molecule and ensemble imaging methods.

Previously, we developed monovalent quantum dot bioimaging probes for tracking the dynamics of transmembrane receptors on live cells (Farlow, 2013). With these probes we measured the diffusion dynamics of Notch revealing slow diffusion ($0.08\mu\text{m}^2/\text{s}$) in comparison to a generic type I transmembrane receptor ($0.29\mu\text{m}^2/\text{s}$), suggesting that Notch is interacting and/or confined prior to activation. Additionally, our static and live cell studies of the receptor on the ensemble level showed spatial organization of Notch at the cell surface on the micron length scale, including specific exclusion

from focal adhesions on the basal membrane. In order to narrow down possible interactions made by Notch, we generated truncated receptor constructs and imaged their spatial distribution and single molecule dynamics in live cells. We found that exclusion from focal adhesions seems to be mediated by the Notch extracellular domain. Single particle tracking revealed that the interactions made by Notch at the cell surface were context dependent, as the diffusion constant of Notch varied with specific truncations to the extracellular domains. Currently, studies are underway to characterize the effects of known Notch pathway regulators on membrane distribution, dynamics, and signal activation.

P472

Board Number: B618

Molecular Anatomy of Early Events in STIM1 Activation; Oligomerization or Conformational Change?.

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A decrease in the luminal ER Ca²⁺ concentration triggers oligomerization and clustering of the ER Ca²⁺ sensor molecule, STIM1, to promote association of its CAD/SOAR domain with the plasma membrane Orai1 channels leading to increased Ca²⁺ influx. A key step in STIM1 activation is the release of the CAD/SOAR domain from the intramolecular clamp that it forms with the first coiled-coil region (CC1, 234-343) of the molecule. Here we used an approach that allowed studying the early molecular events controlling CC1-CAD/SOAR interactions for the first time in intact live cells. We used STIM1 mutants truncated within the CC1 domain and expressed them together with the isolated STIM1 CAD/SOAR domain. We found that the CAD/SOAR domain was bound to ER-localized STIM1(1-343) in quiescent cells but was rapidly released after ER Ca²⁺ depletion and re-associated upon store refilling. The CC1 domain truncated at residue 280 [STIM1(1-280)] was still able to bind the CAD/SOAR domain in a manner dependent on the ER luminal Ca²⁺ concentration. Importantly, CAD/SOAR was not released by artificial oligomerization of a STIM1 (1-343) construct containing FKBP12 in its luminal segment, even though it was released in response to ER Ca²⁺ depletion. Using bimolecular fluorescence complementation of YFP molecules, we also show that STIM1 (1-343) is minimally dimeric. Furthermore, using FRET measurements on a combination of tagged full length STIM1 constructs (with D76A or L251S mutations) and STIM1 molecules only containing the CC1 domain, we show that STIM1 CC1 is able to bind CAD/SOAR in trans. Artificial clustering of either the entire cytosolic domain [STIM1(238-685)] or STIM1(203-685), which lacks the Ca²⁺ sensing modules in the ER lumen, bypasses the early triggering events to fully activate the STIM1 and Orai1 proteins and generate Ca²⁺ influx. The liberated CAD/SOAR domain appears to be the major driver in clustering and activation of the Orai1 Ca²⁺ channels. We conclude, based on these experiments with live cells, that CAD/SOAR is not necessary for STIM1 dimer assembly, but that the release of CAD/SOAR from its CC1 interaction is likely controlled by a conformational change that occurs in STIM1 dimers upon Ca²⁺ unbinding and leads to STIM1-Orai1 clustering.

P473**Board Number: B619****Phosphatidylinositol-3,5-bisphosphate in vacuolar and lysosomal acidification.**S.C. Ho¹, C.H. Choy¹, C.A. Wattson¹, R. Botelho¹;¹Ryerson University, Toronto, ON

Lysosomes and the yeast vacuole are highly acidic and enriched in a variety of acid hydrolases. The acidic environment is required for the proper degradation of cell components and pathogens. Quinacrine, a fluorescent base that readily accumulates in acidic environments, such as the yeast vacuole, fails to accumulate in yeast vacuoles deficient in phosphatidylinositol-3,5-bisphosphate (PtdIns3,5P₂), which suggests that PtdIns3,5P₂ is necessary for acidification. PtdIns3,5P₂ is implicated in a variety of cellular processes such as endolysosomal trafficking, autophagy, and ion channel regulation. PtdIns3,5P₂ is synthesized by the PtdIns5-kinase, PIKfyve, or Fab1 in yeast. However, the role of PtdIns3,5P₂ in vacuolar acidification remains unclear. In addition, the quinacrine assay is not a quantitative measurement of pH. Thus, the actual vacuolar pH in PtdIns(3,5)P₂-deficient vacuoles is not known. Therefore, we developed a fluorimetric assay to measure vacuolar pH using cDCFDA. cDCFDA is a fluorescent base that accumulates in vacuoles irrespective of acidity but its fluorescent intensity is dependent on pH. Our findings indicate that both wild-type and PtdIns(3,5)P₂-deficient vacuoles are acidic, with pH <5.0. In comparison, the vacuolar pH of the V-ATPase mutants, vph1Δ and vph1Δ fab1Δ double mutants were both 6.1. These values were comparable across two background strains, SEY6210 and BY4241. Since these observations were unexpected, we employed a second method based on super-ecliptic pHluorin, a GFP whose fluorescence is very sensitive to acidic pH. Our data showed that the fluorescence signal of pHluorin fused to a methionine transporter, Mup1, is quenched in both wild-type and fab1Δ vacuoles when Mup1 is targeted to the vacuole for degradation. Forcing the alkalinization of the vacuolar pH with an ionophore increased the fluorescent signal of Mup1-pHluorin in both wild-type and PtdIns(3,5)P₂-deficient vacuoles by 5-fold. Overall, we have two independent and quantitative assays showing that vacuoles in fab1Δ cells acidify as well as wild-type cells. Finally, we also measured the lysosomal pH in mammalian cells inhibited for PIKfyve by ratiometric imaging. We labelled lysosomes by endocytosis of FITC-dextran, whose fluorescence intensity is also dependent on pH. The results were consistent with yeast, where lysosomal pH was <5 in control and cells treated with PIKfyve inhibitors, apilimod and MF4. Together, our findings indicates that PtdIns(3,5)P₂ may not be involved in maintaining the steady-state vacuolar/lysosomal pH.

P474**Board Number: B620****EXPRESSION OF GLUCOSE TRANSPORTER 8 IS ASSOCIATED WITH LACTOSE SYNTHESIS IN MAMMARY GLAND OF MICE.**M. Villagran¹, M. Muñoz¹, K. Muñoz¹, I. Ruminot¹, C. Venegas¹, R. Del Pozo¹, L. Mardones¹;¹Faculty of Medicine, Universidad Católica de la SSMA Concepción, CONCEPCION, Chile

Glucose uptake in mammary gland increases suddenly during pregnancy to supply energy for high proliferation rate, and in lactation to support increased metabolic activity for milk synthesis. Glucose transporter 8 (GLUT8) is a class III facilitative glucose transporter located mainly intracellularly. Although this transporter has been located in mammary gland of different species, its physiological role within this gland remains unknown. We hypothesize that GLUT8 is involved in lactose synthesis mediating glucose uptake into Golgi of alveolar cells. To study this issue, we analyzed the temporal expression and the cellular and sub-cellular localization of GLUT8 in murine mammary gland during pregnancy, lactation and early weaning by immunohistochemistry, RT-PCR and western blot. Different cell populations were identified with specific markers: adipocytes with GLUT4, alveolar epithelial cells with cytokeratin 18 (CK18), ductal epithelial cells with cytokeratin 14 (CK14) and mioepithelial cells with smooth muscle actin (SMA). We found GLUT8 transporter colocalized with CK18 and GLUT4 but not with CK14 nor SMA indicating that GLUT8 is constitutively expressed in alveolar epithelial cells and adipocytes but absent in mioepithelial and ductal cells. In alveolar cells, GLUT8 has an intracellular granular pattern shared with lactalbumin. Lactalbumin is an endogen protein of alveolar cells, located in the Golgi, which acts as regulatory subunit of lactose synthase enzymatic complex. Semi-quantitative analysis of peroxidase-stained formalin-fixed tissue samples revealed a progressively increment in GLUT8 expression in alveolar epithelial cells through the pregnancy and the lactation, increasing considerably in early lactation (2 days after birth) and reaching its highest expression at late lactation (10 days). Two days after weaning GLUT8 expression showed fall-down. The highest expression of GLUT8 (late lactation) was associated with the highest percentage of glands stained (80%), percent of cells stained for gland (90%) and intensity observed, meanwhile in virgin mice, only 20% of the glands were marked with less of 20% of cells stained in each gland with the lowest intensity observed. This expression pattern is also observed at mRNA level and in whole protein extracts, where the highest increase was of 10 and 3 fold, respectively. The location of GLUT8 in the Golgi of alveolar cells, its co-location with lactalbumin and its progressive expression through pregnancy suggest its possible association with lactose synthesis enabling glucose entrance to Golgi as a precursor necessary to synthesize this disaccharide.

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P475**Board Number: B621****Identification of a Calsequestrin-1 Mutation in a Human Vacuolar Myopathy.**D. Rossi¹, B. Vezzani¹, L. Galli¹, V. Barone¹, S. Spinozzi¹, V. Del Re¹, A. Gamberucci¹, S. Lorenzini¹, C. Paolini², F. Protasi², C. Reggiani³, V. Sorrentino¹;¹Molecular and Developmental Medicine, University of Siena, Siena, Italy, ²Neuroscienze e Imaging, Università G. d'Annunzio, Chieti, Italy, ³Biomedicine, University of Padova, Padova, Italy

Calsequestrin-1 (CASQ1) is the major calcium binding protein of the sarcoplasmic reticulum (SR) of skeletal muscle cells. It is mainly localized in the junctional domain of the SR where it is part of a quaternary complex, which includes the ryanodine receptor calcium release channel, junctin and triadin. Calsequestrin-1 can modulate Ca²⁺ release by either directly bind the ryanodine receptor and/or by binding to junctin and triadin. We recently identified a D244G mutation in CASQ1 in patients with a myopathy characterized by the presence of vacuoles containing aggregates of SR proteins. The mutation affects a conserved aspartic acid located in one of the high-affinity Ca²⁺ binding sites of CASQ1. We found that muscle fibers from patients carrying the CASQ1 mutation show alterations in the Ca²⁺ release kinetics, thus suggesting that the D244G mutation may alter the intracellular Ca²⁺ signaling in the affected fibers. Interestingly, mutations in the CASQ2 protein identified in patients affected by catecholaminergic polymorphic ventricular tachycardia (CPVT) were shown to alter either Ca²⁺ buffering or Ca²⁺ release properties of cardiac muscle cells and to reduce the ability of calsequestrin to bind junctin and triadin. In order to understand the cellular mechanisms responsible for alterations of Ca²⁺ release kinetics in skeletal muscle cells carrying the D244G mutation, interactions between mutated and wild type CASQ1 and the ryanodine receptor type 1, junctin and triadin are being investigated.

Signaling Receptors (RTKs and GPCRs) 1**P476****Board Number: B623****Optogenetic Control of Class A Orphan G-Protein Coupled Receptors.**M. Morri¹, J. Schwarz¹, H. Janovjak¹;¹IST - Austria, Klosterneuburg, Austria

G-protein coupled receptors (GPCRs) are the largest family of cell membrane receptors and involved in virtually every physiological process. The exact size and diversity of the GPCR superfamily is still unknown and approximately 800 seven transmembrane (7TM) receptors have been identified in the human genome. A large number of 7TM receptors express olfactory receptors (approximately 450 genes), while over 300 genes have functions other than olfaction and activate G-proteins upon stimulation by e.g. hormones, ions, fatty acids or even light. Notably, a considerable number of human 7TM receptors (appx. 150 genes) are considered by The International Union of Basic and Clinical

Pharmacology as orphan GPCRs as their native ligands, signaling pathways and physiological functions are yet to be determined. To understand the signaling function and physiological role of human orphan GPCRs, we combined optogenetics and high-throughput genetic engineering to create light-activated chimeric variants of 90 class A orphan GPCRs. In first experiments, chimeric receptor design and high throughput assays of G-protein activation were validated using well-studied class A GPCRs and their chimeric variants, for which activation by light was compared to activation by ligand. After validation, we used the methodology to test for the ability of all human orphan GPCRs to activate cellular signaling pathways, and to identify their downstream coupling specificity. In several cell types, we detected coupling to one or several G-proteins for a large number of orphan GPCRs. Our work demonstrates that many orphan GPCRs possess the ability to activate cellular signals and that a complete and original library of synthetic receptors can be created for use as optogenetic tools in vitro as well as in vivo.

P477

Board Number: B624

Defining the rules of GPCR-G protein interaction.

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G protein-coupled receptors (GPCRs) are promiscuous signal transducers that bind to and activate multiple downstream G proteins or arrestins to communicate extracellular stimuli into the cell. Dysfunction in GPCR signaling is implicated in a wide range of disease states, including cardiac myopathies, neurodegenerative disorders, psychoses, retinal degeneration, and cancer. Despite large-scale efforts, the rules of GPCR-G protein interaction remain undefined. To complement the existing FRET/BRET methodologies, we have developed a modular system to control and visualize GPCR-G protein interactions. Using this technology, we characterized the basal association and functional interaction between the prototypical GPCR opsin, four distinct adrenergic receptors ($\alpha 1$, $\alpha 2$, $\beta 2$, and $\beta 3$ -AR), and adenosine type 1 receptor with Gs, Gi, and Gq. In general, while GPCRs bind G proteins with varying strengths, Gs always associates stronger than Gi or Gq. Despite binding preferences, GPCRs do maintain an inherent propensity for activating their canonical G protein pathway. Interestingly, the strength of the GPCR-G protein binding does not correlate with the efficacy of pathway activation. The functional implications of these observations, sequestration of G proteins, regulation by RGS proteins, and differential signaling by GPCRs are presented. Together, our studies provide a blueprint to broadly interpret GPCR-G protein interactions in the presence and absence of ligands.

P478**Board Number: B625****The increasing complexity of Wnt signaling at the cell surface.**A. Person¹, W. Xiong¹, K. Shields¹, B. Burroughs¹, C. Howe¹, V. Kalabokis¹;¹Bio-Techne, Minneapolis, MN

The complexity of Wnt signaling is apparent due to the large number of ligands, receptors, and secreted inhibitors that have been implicated in Wnt signaling. Recently, newly described adult stem cell markers, LGR5 and TROY, were shown to play prominent roles in the increasingly complex regulation of Wnt signaling at the cell surface. Over ten years ago, the secreted proteins, R-Spondins, were shown to enhance Wnt signal transduction, but only recently was the mechanism of R-Spondin-mediated Wnt activation uncovered. R-Spondins are now known to bind to the Leucine-rich repeat-containing G-protein coupled Receptors (LGR 4-6), the transmembrane ubiquitin ligase Ring Finger 43 (RNF43), and the related ubiquitin ligase Zinc/Ring Finger 3 (ZNRF3). RNF43 and ZNRF3 both negatively regulate Wnt signaling by ubiquitinating Frizzled receptors targeting them for internalization and degradation. R-Spondins bind to both LGR4-6 and RNF43/ZNRF3 with this complex formation initiating endosomal internalization of the negative Wnt signaling regulators RNF43/ZNRF3. In addition, the tumor necrosis factor receptor superfamily member 19 (TNFRSF19 or TROY) is a membrane protein that binds LGR receptors and negatively regulates Wnt signaling with an incompletely understood mechanism. R&D Systems is in a unique position to comprehensively investigate the physical interactions and the functional significance of these new cell surface Wnt pathway regulatory proteins as we have generated all of the necessary purified recombinant proteins for this analysis. Recombinant extracellular domain LGR4-6 proteins all bind R-Spondins, but show preferential binding to different R-Spondin family members. R-Spondin proteins bind to the extracellular domain of RNF43/ZNRF3 with high affinity, and the extracellular domain of TROY also interacts with LGR5. In Wnt reporter assays, addition of LGR4 and 6 extracellular domain proteins robustly enhance Wnt3a activity in HEK293 cells. Our data demonstrate that addition of the LGR proteins is sufficient to enhance R-Spondin mediated stimulation of Wnt signaling by inhibiting the negative regulators RNF43/ZNRF3. Interestingly, we also detect inhibition of R-Spondin activity when LGR4-6 proteins are added to Wnt reporter cells in the context of saturating doses of R-Spondins. Finally, addition of the RNF43 and ZNRF3 proteins inhibit R-Spondin-enhanced Wnt reporter activation. We present new binding and functional data describing these new Wnt pathway-related recombinant proteins, and we also describe the Wnt assay systems that have allowed us to better understand these complex new regulators of Wnt signaling.

P479**Board Number: B626****Evolutionarily Conserved Coupling of Adaptive and Excitable Networks Mediates Eukaryotic Chemotaxis.**C. Huang¹, P. Devreotes², P. Iglesias³, M. Tang⁴, M. Wang¹, C. Shi¹;¹Johns Hopkins University, Baltimore, MD, ²Department of Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD, ³Electrical and Computer Engineering, Johns Hopkins University, Baltimore, MD, ⁴Johns Hopkins University School of Medicine, Baltimore, MD

Numerous models have been proposed to explain the remarkable ability of chemotactic cells to sense and migrate toward extremely shallow chemoattractant gradients independently of the ambient concentration. Recent studies indicate that the stochastic activity of an excitable signal transduction network involving Ras, PI3K, and Rac acts as a pacemaker that controls the cytoskeleton to drive motility. Here we show that blocking multiple signal transduction pathways simultaneously abrogated chemoattractant-induced actin polymerization and chemotactic motility but not directional sensing. We carried out experiments to distinguish the various models of gradient sensing in migrating cells. First, signaling activity was strongly suppressed toward the low side of cells in a gradient or following sudden removal of uniform chemoattractant. Second, signaling activities displayed a rapid shut off and, with stimulation of increasing duration, a slower adaptation during which responsiveness to subsequent test stimuli declined. Simulations of existing classes of models indicated that these observations can only be explained by the coupling between an adaptive module and an excitable network. Moreover, stimulation of cells lacking G-protein function suppresses downstream activities, while constitutive G-protein activation induced persistent responses. This indicates that chemoattractant sensing is mediated by a balance between G-protein dependent and independent signals. The salient features of the coupling between adaptive and excitable networks were observed for the chemoattractants cAMP and folic acid in *Dictyostelium* as well as fMLP in human neutrophils, suggesting an evolutionarily conserved mechanism for eukaryotic chemotaxis.

P480**Board Number: B627****An oncogenic c-KIT mutant can bypass SLAP-mediated negative regulation by phosphorylating SLAP.**J.U. Kazi¹, J. Sun¹, L. Rönnstrand¹;¹Translational Cancer Research, Lund University, Lund, Sweden

The stem cell factor receptor, c-KIT has been implicated in various malignancies including mastocytosis, some subtypes of acute myeloid leukemia, small cell lung carcinoma, malignant melanoma, colorectal cancer and gastrointestinal stromal tumors. Within the more than 500 mutations that have been found, the D816V mutation is the most abundant oncogenic mutation of c-KIT in human malignancies. Under

normal condition the receptor signaling is tightly regulated by regulatory proteins such as phosphatases, ubiquitin ligases and adapters or scaffold proteins. The SRC-like-adaptor protein (SLAP) has been shown to be a negative regulator of mitogenic signaling. In many cases SLAP expression overlaps with c-KIT expression and therefore we aimed to determine whether SLAP is involved in regulation of c-KIT downstream signaling. We observed that SLAP associates with both wild type c-KIT and the oncogenic mutant KIT-D816V. The SH2 domain of SLAP is involved in association with c-KIT through phosphotyrosine residues. SLAP induces ubiquitination and degradation of c-KIT resulting in negative regulation of downstream signaling. Unlike wild type c-KIT, SLAP did not block c-KIT-D816V-mediated phosphorylation of downstream signaling mediators such as AKT and ERK1/2. To understand the mechanism how c-KIT-D816V escapes SLAP-mediated controls, we studied localization and posttranslational modifications of SLAP in c-KIT-D816V expressing cells. We observed that c-KIT-D816V, but not wild type c-KIT, specifically phosphorylates SLAP on Y120, Y258 and Y273 residues. A mutant lacking the three phosphorylation sites can restore SLAP regulation on oncogenic c-KIT-D816V. Taken together, our data suggest that SLAP is an important regulator of c-KIT signaling and oncogenic c-KIT-D816V can bypass normal cellular regulation by phosphorylating SLAP and thereby enhanced oncogenic signaling.

P481

Board Number: B628

PI 3 kinase is indispensable for oncogenic signal transduction by the V560D mutant of c-Kit.

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Oncogenic mutations of c-Kit are often identified in mastocytosis, gastrointestinal stromal tumors (GISTs) and acute myeloid leukemia (AML). The activation mechanism of the most often occurring mutation, D816V in exon 17 of c-Kit, has been well studied while other mutations remain fairly uncharacterized in this respect. In this study, we show that the constitutive activity of the exon 11 mutant V560D is weaker than the D816V mutant except that the phosphorylation of the Src family kinase binding site, Tyr568, is similar in both c-Kit mutants. The ligand for c-Kit, stem cell factor (SCF), induced phosphorylation of downstream signaling proteins, such as Akt and Erk, in c-Kit/V560D expressing cells was stronger than that in c-Kit/D816V expressing cells. Although cells expressing c-Kit/V560D showed increased ligand-independent proliferation and survival compared to wild-type c-Kit expressing cells, these biological effects were less than c-Kit/D816V expressing cells. In contrast to cells expressing wild-type c-Kit, cells expressing c-Kit/V560D are independent of Src family kinases for downstream signaling. However, this independence of Src family kinases is not due to a Src-like kinase activity of the mutant receptor that c-Kit-D816V holds. Blockage of the association of PI3 kinase with c-Kit/V560D by point mutation, but not inhibition of PI3-kinase with pharmacological inhibitors, totally blocked ligand-independent activation of the receptor, suggesting a key role of the non-lipid kinase activity of PI3 kinase in c-Kit/V560D mediated oncogenic signal transduction. Thus, PI3 kinase is an

attractive treatment target in c-Kit mutations induced malignancies independent of its lipid kinase activity.

P482

Board Number: B629

Palmitoylation of EGFR attenuates receptor signaling.

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Cellular signaling pathways important for tumor initiation and progression are regulated by a variety of well-studied post-translational modifications including phosphorylation, ubiquitination and acetylation. Recently, the reversible lipid modification; palmitoylation, has been demonstrated to control the localization and activity of a variety of proteins, thereby serving as a novel modification that has the ability to alter signaling pathways in cancer.

Palmitoylation is regulated by two classes of enzymes, the DHHC domain containing protein acyl-transferases (PAT) which mediate the addition of palmitate to target substrates, and the acyl-protein thioesterases (APT) which remove palmitate from proteins. Twenty-three DHHC PATs have been identified in mammalian cells and alterations in the expression and function of several PATs have been observed in neurodegenerative disorders and diseases such as cancer. Analysis of The Cancer Genome Atlas reveals a loss of expression of the PAT DHHC20 in a subset of breast, lung and prostate tumors suggesting a potential function for DHHC20 in cancer.

Using MDA-MB231, triple-negative breast cancer cells, we have determined that knockdown of DHHC20 increases chemotaxis which is blocked by the EGFR inhibitor Gefitinib. Closer examination of EGFR signaling in DHHC20 knockdown cells revealed elevated EGFR expression and amplified EGF-induced activation of EGFR and AKT. The expression and activation of EGFR are regulated by receptor mediated endocytosis and lysosomal degradation. Knockdown of DHHC20 impairs the endocytic trafficking of EGFR to lysosomes in response to EGF, which may contribute to the sustained signaling.

The specificity of DHHC enzyme-substrate pairs has not been well studied and little is known about specific targets of DHHC20. Our studies have identified EGFR as a novel substrate of DHHC20. Blocking palmitoylation by mutating intracellular cysteines on EGFR revealed putative palmitoylation sites that activate EGFR independent of EGF. These findings support the importance of palmitoylation in regulating EGFR activation. Finally, inhibiting EGFR activity is critical for treating cancers in which EGFR is highly expressed or constitutively activated. We found that pharmacological inhibition of protein depalmitoylation with the APT1 inhibitor Palmostatin B attenuates EGFR activation in a dose dependent manner, suggesting a potential treatment strategy to regulate EGFR signaling in cancer. Taken together, our findings identify palmitoylation as a novel modification of EGFR that regulates receptor activation. Maintaining EGFR in its palmitoylated form may serve as an innovative treatment approach to control EGFR activation in cancer.

P483**Board Number: B630****Na,K-ATPase function modulates EGFR signaling.**A. Litan¹, S. Lee¹, D. Wolle¹, Z. Li¹, S. Yoo¹, S.A. Langhans¹;¹A I duPont Hosp Children, Wilmington, DE

EGF regulates cell proliferation and migration by triggering molecular cascades, including Erk and PI3K/Akt signaling, that regulate cytoskeletal reorganization, cell cycle progression and gene expression. Aberrant expression/activation of EGFR is found in multiple human cancers, including medulloblastoma, the most prevalent pediatric brain cancer. Termination of EGF signaling is tightly regulated by endocytosis of the activated EGF receptor (EGFR) followed by trafficking of the receptor-ligand complex to specific endocytic compartments. Trafficking of EGFR relies on microtubule tracks that transport cargo vesicles to their intermediate and final destinations and can be modulated through posttranslational modification of tubulin. Using cultured medulloblastoma cells, we found that EGF-induced sodium influx regulates EGFR trafficking through increased microtubule acetylation. Increased sodium influx induced by sodium ionophores mimicked the EGF-induced effects on EGFR trafficking through histone deacetylase (HDAC) 6 inactivation and accumulation of acetylated tubulin. Furthermore, the cardiac glycoside, ouabain which specifically blocks the pump activity of Na,K-ATPase induced tubulin acetylation, suggesting that intracellular sodium homeostasis can modulate tubulin acetylation and may fine-tune tubulin function. Consistent with a role of intracellular sodium homeostasis regulating EGFR signaling, ouabain treatment modulated EGF-induced Erk1/2 and Akt signaling, blocked EGF-induced formation of actin stress fibers and cell motility, and modulated response to EGFR tyrosine kinase inhibitors. Together our data suggest that an increase in intracellular sodium is a component of an auto-regulatory loop that regulates termination of EGFR signaling through trafficking of the EGF-EGFR complex and includes HDAC6 and tubulin acetylation.

P484**Board Number: B631****The secreted LPR-1 facilitates LIN-3/EGF signaling during the development of the excretory system.**P. PU¹, M.V. Sundaram¹;¹Department of Genetics, University of Pennsylvania, Philadelphia, PA

LPR-1 facilitates LIN-3/EGF signaling during the development of the *C.elegans* excretory system Pu Pu, Meera V. Sundaram

The activity of signaling ligands such as Epidermal Growth Factor (EGF) must be tightly controlled in space and time to ensure that signaling is restricted to appropriate target cells. In the *C.elegans* excretory (renal like) system, the EGF/Ras/Erk signaling pathway plays multiple roles in specifying the

duct versus the pore cell fate and maintaining organ architecture. LPR-1 belongs to the large lipocalin family, which is a group of small secreted proteins functioning as carriers for lipophilic cargos. *lpr-1* loss-of-function mutants showed a highly penetrant L1 lethal excretory phenotype due to discontinuous lumen between the duct and pore, whereas this lethality was significantly suppressed by overexpressing a *lin-3*/EGF genomic fragment or by hyperactivating the downstream LET-60/Ras signaling pathway within the developing excretory duct and pore. By alternative splicing, *lin-3* generates at least 4 protein isoforms. In order to examine which one or more isoforms of LIN-3/EGF could function in the excretory system and rescue the *lpr-1* mutant excretory defect, 4 different LIN-3 isoforms were expressed under the control of its own promoter. All 4 LIN-3 isoforms could function in the excretory system and rescue *lin-3* and *lpr-1* mutant lethality, although at different efficiencies, with the predominant embryonic isoform showing relatively low signaling efficiency. Furthermore, *lpr-1* loss could suppress the excretory pore-to-duct fate transformation of *lin-15* SynMuv mutants, which have slightly elevated levels of *lin-3* expression. These epistasis results suggest that during excretory system development, LPR-1 facilitates efficient LIN-3/EGF signaling.

P485

Board Number: B632

Induced proinflammatory signaling limits proliferative response to FGF.

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We found that transient FGF1 or FGF2 stimulation of various cell types results in the novel phenomenon of “FGF memory” – a sustained blockage of efficient proliferative response to FGF, PDGF or EGF. FGF memory establishment requires HDAC activity, indicating its epigenetic character. FGF treatment stimulates the proinflammatory NFκB signaling, which is also critical for FGF memory formation. The search of potential FGF-induced mediators of FGF memory revealed that FGF stimulates a sustained expression of the inflammatory cytokine IL1α. Similarly to FGF, transient cell treatment with recombinant IL1α inhibits the proliferative response to further FGF stimulation, but does not prevent the FGF receptor-mediated signaling. Interestingly, cells pretreated with FGF1 or IL1α exhibit an enhanced restructuring of actin cytoskeleton and migration in response to FGF stimulation. IRAP, a specific inhibitor of IL1 receptor prevents the formation of FGF memory and rescues an efficient proliferative response to FGF restimulation. Thus, FGF1 memory is mediated by proinflammatory IL1 signaling. It may play a role in the limitation of proliferative response to tissue damage and prevention of wound-induced hyperplasia.

P486**Board Number: B633****Regulation of the "molecular brake" region in FGFR2 kinase.**J.M. Karp¹, D. Cowburn¹;¹Albert Einstein College of Medicine, Bronx, NY

Unregulated activity of tyrosine kinases (TK) is responsible for numerous developmental musculoskeletal diseases and cancers. The regulatory mechanisms of TKs and how they are affected by point mutations are still in need of elucidation. Fibroblast growth factor receptor (FGFR) kinase is a TK whose regulatory element, the "molecular brake," is thought to prevent constitutive activation of the receptor, despite not being near the kinase's active site. Germline mutations in this "molecular brake" region in FGFR cause musculoskeletal disorders such as craniosynostosis and dwarfism, while somatic mutations can lead to uninhibited cell growth, causing an array of cancers. Furthermore, it is thought that the majority of pathogenic activating mutations in protein kinases are in "molecular brake"-like regions. However, the mechanism through which the "molecular brake" of FGFR kinase is disengaged, allowing for signal transduction, is still unknown. Using path-based molecular dynamics (MD) simulations, we have shown that the inward motion of the kinase's activation loop upon tyrosine phosphorylation is correlated with disengagement of the "molecular brake." Results of the string method in collective variables, which finds the minimum free energy path connecting the loop-out and loop-in states, indicate found that inward motion of loop occurs within 1 frame of "molecular brake" disengagement. Furthermore, umbrella sampling shows that in loop-out conformations, the disengaged state of the "molecular brake" is less stable, while in loop-in conformations, the disengaged state becomes more stable. Our results suggest that the "molecular brake" is allosterically controlled by the activation loop motion. An understanding of the "molecular brake" disengagement mechanism will provide avenues for targeted therapies to counteract pathologic over-activation of FGFR kinase leading to uncontrolled signal transduction and uninhibited cell growth.

P487**Board Number: B634****Essential roles of Crk and CrkL in fibroblast growth.**T. Park¹, T. Curran¹;¹Department of Pathology and Laboratory Medicine, The Children's Hospital of Philadelphia Research Institute, Philadelphia, PA

Cytosolic proteins containing SH2 and SH3 domains, such as Crk and Crk-like (CrkL), are broadly expressed adaptors that interact with a variety of proteins to fulfill key roles in signal transduction pathways triggered by activation of receptor and non-receptor tyrosine kinases. Crk and CrkL are similar to each other in structure and biochemical function, and they provide both overlapping and distinct biological roles during development. We took a systematic approach to investigate Crk family functions at the cellular level by generating conditional knockout systems for ablation of Crk and CrkL in cultured

fibroblasts. Previously, we used lentiviral infection to induce ablation of Crk and CrkL in slowly growing fibroblasts and demonstrated that Crk and CrkL play critical roles in cell structure and motility by maintaining cytoskeletal integrity. Here we transfected rapidly dividing fibroblasts with modified RNAs synthesized in vitro using the Neon transfection system to study roles of Crk and CrkL in cell growth. Most cells became positive for green fluorescence 5 hours after transfection with GFP RNA, suggesting that RNAs are efficiently translated into proteins upon transfection. Loss of Crk and CrkL induced by Cre RNA transfection blocked cell proliferation in fibroblasts immortalized either by T antigen transfection or by culturing according to the 3T3 protocol. Western blot analyses indicated that transfection of Crk/CrkL double floxed cells with Cre RNA blocked expression of the three Crk family proteins, CrkII, CrkI and CrkL. In addition, expression of beta-actin protein and phosphorylation of both p130Cas and Akt were reduced in the absence of Crk and CrkL. Immunofluorescence staining revealed that the cytoplasm and nucleus became smaller in the absence of Crk and CrkL. Ablation of Crk or CrkL alone conferred a much more modest inhibition of cell proliferation suggesting that Crk and CrkL play overlapping functions in cell growth. Taken together, our results suggest that Crk and CrkL play essential roles in cell growth.

P488

Board Number: B635

The role of EphA2 in regulating HGF-induced epithelial morphogenesis in three-dimensional culture.

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EphA2, a member of Eph family receptor tyrosine kinases, is overexpressed in various cancers derived from epithelial cells and promotes cancer cell migration and invasion. In contrast, its molecular mechanisms and functional roles in epithelial morphogenesis remain unclear. In this study, we show that EphA2 promotes extension formation, the first step of tubulogenesis, in three-dimensional cultured MDCK cysts in response to hepatocyte growth factor (HGF) stimulation. Interestingly, we also found that serine 897 phosphorylation, but not tyrosine kinase activity, of EphA2 is necessary for this morphogenesis. Moreover, ephrinA1 stimulation blocks HGF-induced extension formation. These observations suggest that ligand-independent activity of EphA2 is essential for epithelial morphogenesis.

P489**Board Number: B636****Sphingosine 1-Phosphate Mediates Epithelial-Mesenchymal Transition in Breast Cancer.**W. Wang¹, D. Herr¹;¹Department of Pharmacology, National University of Singapore, Singapore, Singapore

Epithelial-mesenchymal transition (EMT) is an essential process implicated in cancer metastasis, leading to cancer progression. Signaling pathways contributing to EMT are largely unknown. Sphingosine 1-phosphate (S1P), a bioactive lipid mediator, mainly exerts its effects by binding to a family of G protein-coupled receptors (GPCRs), namely S1P1-5. S1P signaling has been reported to regulate cell migration and motility in cancers; nevertheless, its role in EMT is not well established. Here we show that S1P signaling mediates EMT process via transcription factor Slug, which is one of the EMT master regulators. S1P treatment, particularly short term, can significantly up-regulate the level of Slug in breast cancer cell lines. A group of available ligands for S1P receptors have been validated using a novel tool (TGF α shedding assay) for the first time for their antagonistic and/or agonistic activities, as well as their specificity and potency. By pharmacological and genetic approaches, S1P2 and S1P3 are identified as two main receptors responsible for this signaling pathway, and most likely their downstream effectors Rho and Rac are also involved. Preliminary results suggest that S1P signaling up-regulates Slug expression level by promoting its transcription. Besides, efforts have been made to investigate the crosstalk between S1P-induced EMT and other known EMT pathways, such as TGF beta and EGFR/HER2. Taken together, our findings demonstrate that S1P signaling immediately up-regulates Slug level through S1P2 and S1P3, contributing to the initial stage of EMT. This study may provide new insights for therapeutic approaches in the early stage of cancer metastasis.

P490**Board Number: B637****Profibrotic upregulation of GLUT1 and Hexokinase 2 by transforming growth factor beta requires autocrine receptor tyrosine kinase mediation.**M. Andrianifahanana¹, X. Yin¹, Y. Wang¹, E.B. Leof¹;¹Thoracic Disease Research Unit, Division of Pulmonary and Critical Care Medicine, Departments of Internal Medicine and Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN

Defining the exact mechanisms by which transforming growth factor β (TGF β) exerts its profibrotic activity is critical for understanding the molecular basis as well as developing viable therapeutic strategies for disorders with a major fibroproliferative component, such as fibrosis and desmoplastic cancers. Here, we report that TGF β causes fibroblast cells to coordinately upregulate expression of the facilitated glucose transporter 1 (GLUT1/SLC2A1) and its downstream effector, hexokinase 2 (HK2), both at the mRNA and protein levels. Using pharmacological and genetic approaches we demonstrate that

GLUT1/HK2 induction occurs via the canonical Smad2/3 pathway and requires autocrine activation of the receptor tyrosine kinases, platelet-derived (PDGFR) and epidermal (EGFR/ErbB) growth factor receptors, whereby the MAPK/ERK (mitogen-activated protein kinase/extracellular signal-regulated kinase) module serves as a common effector. Of note, inhibition of GLUT1 activity is shown to prevent TGF β -driven anchorage-independent growth of fibroblasts on soft agar. Together, our results provide new insights into the mechanisms controlling upregulation of the glucose metabolic pathway and its role in profibrotic TGF β signaling. These findings may shed light on mechanisms associated with the pathogenesis of fibroproliferative diseases and contribute to the identification of novel therapeutic targets.

P491

Board Number: B638

Fc ϵ RI Signal Propagation is Regulated Through Transient Binding of Syk.

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The high affinity IgE receptor, Fc ϵ RI, serves as the primary immunoreceptor on mast cells and basophils. Crosslinking of IgE-bound Fc ϵ RI via multivalent antigen induces phosphorylation of the immunoreceptor tyrosine-based activation motifs (ITAMs) followed by recruitment of the kinase Syk and further signal propagation. While the sequence of these signaling events has been well studied using biochemical techniques, the biophysical mechanisms that regulate Fc ϵ RI signaling are unclear. Using two-color single molecule imaging in live cells, we quantify how Fc ϵ RI aggregation influences Syk recruitment and mobility. We imaged the basal surface of RBL-2H3 cells expressing mNeon-Syk using TIRF microscopy and upon crosslinking of Fc ϵ RI observed an increase in the membrane localization of mNeon-Syk. Interestingly, while ensemble measurements showed that Fc ϵ RI and mNeon-Syk were co-localized over minutes, single molecule imaging revealed that mNeon-Syk clusters are in fact maintained through a continuous exchange of transiently bound mNeon-Syk. We quantify the residency time of individual mNeon-Syk molecules at Fc ϵ RI aggregates and explore the relationship between Fc ϵ RI aggregate size and Syk residency time. Previously, it has been shown that high concentrations of stimulating cross-linking antigen induce a drastic reduction in Fc ϵ RI mobility. However, when antigen is added at low concentrations, low valency, or presented from a mobile bilayer, this mobility change is less substantial. We use single particle tracking to characterize the mobility of mNeon-Syk and find a similar antigen dependence on mobility. We show that at high concentrations of crosslinking antigen, Syk is highly immobilized at the membrane, yet when antigen is presented at lower concentrations or from a mobile bilayer Syk remains mobile at the membrane while still exhibiting an increased residency time. These results demonstrate that mobile Fc ϵ RI are capable of recruiting Syk and that receptor immobilization is not a requirement for signaling.

P492**Board Number: B639****Autocrine Selection of Novel GABABR Agonists from Intracellular Combinatorial Libraries.**R.E. Turn¹, E. Sturchler², M. Stringhini², C. Rader², P. McDonald²;¹Harriet L. Wilkes Honors Coll, Jupiter, FL, ²Molecular Therapeutics Department, Scripps Research Institute, Jupiter, FL

Dysfunction of GABAB receptor (GABABR)-mediated synaptic transmission underlies various nervous system disorders including epilepsy, depression, schizophrenia, and addiction. Currently, only one GABABR orthosteric ligand namely Baclofen, is in clinical use. However, side effects such as sedation and tolerance limit its use. GABABRs are G-protein coupled receptors that have been shown to mediate their effects via multiple signaling pathways. Some receptor ligands have been shown to possess multiple efficacies depending on the specific down-stream signal transduction pathway being analyzed. This diversity is believed to be the result of conformational changes induced in the GPCR that are ligand-specific, a phenomenon known as 'functional-selectivity'. Hence, a dissociation of the therapeutic effects from the side effects of GABABR activation may be achievable with more pathway-selective GABABR drugs. In this study, we aim to identify novel positive modulators of GABABR by screening large combinatorial antibody libraries using purified extracellular domain of GABABR. 'Hits' identified in this primary screen will be further investigated in three distinct cell-based functional assays that monitor; 1) cAMP production, 2) intracellular calcium mobilization, and 3) MAP Kinase activation, using a GABABR-expressing CHO cell line, in an attempt to identify those that exhibit functional selectivity.

P493**Board Number: B640****Gene knockouts of two different constitutive GPCR activities produce opposite changes in the excitability of the unicellular ciliate *Tetrahymena thermophila*.**T.M. Hennessey¹, D. Zou¹;¹Biological Sciences, University at Buffalo, Amherst, NY

Tetrahymena are eukaryotic unicells that can move either towards an attractant or away from a repellent by modifying their swimming behaviors. These responses rely on alterations in the basal rate of changes in swimming direction. In bacterial chemotaxis, these changes are called "tumbles" but in *Tetrahymena*, we refer to it as PDC (the percent of cells showing direction changes). In general, attractants decrease basal PDC (allowing them to swim straighter towards the attractant) while repellents increase PDC (to send them in a new, random direction). For well regulated changes in PDC, there should be a relatively high basal PDC so that both increases and decreases are possible. What sets this basal PDC? How can stimuli regulate this PDC to cause changes in swimming behavior? The objective of our work is to try to answer these questions. Our current model (Lampert et al., 2011) is

that basal PDC is kept high in wild type by the constitutive activity of a pertussis-sensitive GPCR (*gpcr6*). This may act by modulating either Ca^{++} channel activity or by Ca^{++} regulation. Some attractants may work as inverse agonists to decrease this GPCR effect, decrease basal PDC and result in chemoattraction. Consistent with this model, we have described a GPCR knockout mutant in *Tetrahymena*, called G6 (Lampert et al., 2011), which has a low basal PDC and an inability to respond to chemoattractants because it can't lower the PDC further. It also under-responds to strong depolarizing conditions like high K^+ , possibly because of its effects on Ca^{++} channel activity. We have constructed another putative GPCR knockout in *Tetrahymena*, called G3, which dramatically over-responds to high K^+ . Analyses of the various behavioral responses of mutants and wild type and their effects on internal Ca^{++} concentrations suggest that the constitutive activity of the wild type GPCR3 gene product (*gpcr3*) may affect the voltage-dependent ciliary Ca^{++} channels to put them in a state that is more "reluctant" (Bean, 1989) to open and establish the basal PDC. We propose that the *gpcr3* knockout mutation puts the Ca^{++} channels in a more "willing" state and makes the G3 mutant more excitable. Together with the results from the G6 mutant, this suggests that the basal PDC is affected by both *gpcr3* and *gpcr6* to regulate the PDC. Attractants and repellents may act as "inverse agonists" to regulate the basal PDC through their effects on these GPCRs and their related Ca^{++} channel activities.

P494

Board Number: B641

Role of RKTG, a novel Golgi localized receptor, in Gbetagamma regulated transport of secretory proteins from the Golgi to the Plasma Membrane.

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Recent studies have revealed a role for G protein betagamma signaling at the Golgi via protein kinase D (PKD) regulating fission of plasma membrane (PM) destined secretory vesicles from the trans Golgi network (TGN). The mechanism by which the Gbetagamma subunits are recruited to and activated at the Golgi in this process remains elusive. Recent studies reveal that a newly characterized Golgi localized, membrane protein belonging to the Progestin and AdipoQ Receptor (PAQR) family, RKTG (Raf Kinase Trapping to the Golgi), interacts with the Gbeta subunit and sequesters Gbetagamma to the Golgi thereby regulating the functions of Gbetagamma. Here we show that over expression of RKTG causes vesiculation of the Golgi, while a Gbeta binding mutant of RKTG does not cause vesiculation. Also, the C-terminal fragment of GRK2 (GRK2ct), which interacts with, and inhibits Gbetagamma signaling, and Gallein, a small molecule inhibitor of Gbetagamma, were both able to inhibit Golgi vesiculation in the presence of RKTG. Furthermore, a dominant negative form of PKD (PKD-DN) and a pharmacological inhibitor of PKD, Go6976, also inhibited RKTG mediated vesiculation of the Golgi. Collectively, these results reveal a novel role for the newly characterized, Golgi localized RKTG receptor, in regulating Gbetagamma at the Golgi, thus controlling Golgi to PM protein transport via the Gbetagamma-PKD signaling pathway.

Signaling Scaffolds and Complexes

P495

Board Number: B642

Interaction of JLP with Plk1 recruits FoxK1 to interact and form a ternary complex.

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JLP (JNK associated Leucine zipper protein) is a scaffolding protein, which has been shown to interact with and activate JNK/p38MAPK pathway. Its interaction with various signaling proteins is associated with coordinated regulation of cellular process such as endocytosis, motility, neurite outgrowth, cell proliferation and apoptosis. Here we identified a mitotic Serine/Threonine kinase, Polo like kinase 1 (Plk1), as a novel interaction partner of JLP through a mass spectrometry based approach. We show that the N-terminal domain of JLP interacts with the polo-box domain (PBD) of Plk1 in a phosphorylation-dependent manner. Our results indicate that, JLP is phospho-primed on Thr 351 residue on its N-terminus, which is recognized by the PBD of Plk1 leading to phosphorylation of JLP at additional sites. Moreover, treatment of cells with the Plk1 inhibitor, BI2536 affects the interaction demonstrating the importance of Plk1 kinase activity in this process. Since JLP is a scaffolding protein that recruits proteins to mediate specific cell signaling events, we hypothesized that the interaction of JLP with Plk1 might result in the recruitment of other proteins to this complex. To test this hypothesis, we carried out SILAC labeling of proteins in mitotic cells in the presence or absence of BI2536. Through mass-spectrometry we identified the transcription factor, FoxK1 as a Plk1-dependent JLP-interacting protein. Furthermore, we show that JLP, Plk1 and FoxK1 form a ternary complex, which occurs only during mitosis. Knockdown of Plk1 and not JLP, affected the interaction between JLP and FoxK1 indicating that the formation of the ternary complex is dependent on Plk1. FoxK1 has been previously characterized as a transcriptional repressor of cyclin dependent kinase inhibitor, p21/WAF1. We observed that knockdown of JLP in U2OS cells results in increased protein levels of FoxK1 and a reduction of p21 expression. Moreover, immunofluorescence studies in asynchronous cells showed that FoxK1 is excluded from the nucleus during mitosis. Based on our observations, we propose that formation of the ternary complex between JLP, Plk1 and FoxK1 regulates the stability and/or localization of FoxK1.

P496**Board Number: B643****The XB130/Tks5 Adaptor Protein Interaction Regulates Src-mediated Signal Transduction.**S. Moodley^{1,2}, M. Liu^{1,2};¹Institute of Medical Science, University of Toronto, Toronto, ON, ²Latner Thoracic Surgery Research Laboratories, Toronto General Research Institute, University Health Network, Toronto, ON

Adaptor proteins are signal transduction organizers that coordinate the formation of molecular complexes and translocation of molecules to relay signals from the extracellular environment to intracellular targets. The adaptor protein, XB130 regulates cell growth, survival and migration. Yeast-2 hybrid screening revealed that XB130 interacts with another adaptor protein, Tks5, which is highly involved in cell migration. We hypothesize that XB130 and Tks5 may act in conjunction to recruit and translocate proteins to initiate signal transduction cascades, leading to regulation of cell growth, survival or motility. Using co-immunofluorescence microscopy, XB130 and Tks5 were detected in the cytoplasm and peri-nuclear region of BEAS-2B human bronchial epithelial cells. Moreover, co-immunoprecipitation showed that XB130 and Tks5 interact endogenously and form a complex with Src-tyrosine kinase. Tks5 interacts with XB130 by binding to polyproline rich motifs in the N-terminal of XB130 and conversely, XB130 binds to the fifth SH3 domain of Tks5, as detected by immunoprecipitation of overexpressed XB130 deletion mutants and GST fusion protein pulldown of XB130 single amino acid substitution mutants or Tks5 SH3 domains. Cell motility studies showed that siRNA downregulation of XB130 inhibits lateral cell migration in a wound healing assay, whereas, downregulation of Tks5 inhibits cell invasion in a gelatin degradation assay. However, cell growth and survival studies showed that downregulation of XB130, Tks5 or both XB130 and Tks5 reduced cell proliferation, as assessed by BrdU incorporation, resulting in accumulation of cells in the G1 phase of the cell cycle and increased caspase 3 activity, as well as, apoptotic cell death, as assessed by PI/Annexin V flow cytometry. Interestingly, cell proliferation and survival were increased by overexpression of XB130 or Tks5 but decreased by overexpression of the XB130/Tks5 binding site mutants; XB130 deleted N-terminal and Tks5 W1108A fifth SH3 domain. Furthermore, overexpression of XB130 and Tks5 with Src enhances Src activation and total cell protein tyrosine phosphorylation but downregulation of XB130, Tks5 or both XB130 and Tks5 inhibited serum and growth factor-induced Src-activation. Our results suggest that XB130 and Tks5 interact endogenously with Src to form a putative protein complex to promote signal transduction. Moreover, the XB130/Tks5 interaction is required to mediate serum and growth factor-induced Src-activation, thereby promoting cell proliferation and survival, in lung epithelial cells.

P497**Board Number: B644****Screening and identification of dissociative inhibitors of ERK MAP kinase signaling pathway.**J. Ha¹, S. Park¹;¹Seoul National University, Seoul, Korea

The ERK mitogen-activated protein kinase (MAPK) pathway plays an essential role in the transduction of developmental and growth signals. The pathway involves kinases which relay phosphorylation to activate downstream kinases or effectors, and scaffold proteins which tether kinases to form a signaling complex. Because of its key function in cell proliferation, misregulation of the ERK pathway often results in uncontrolled cell growth that eventually leads to cancerous morphology changes. Many therapeutic approaches have targeted proteins in the pathway including ATP-competitive inhibitors to inactivate the enzymatic activity of kinases. In this study we attempt to suppress the ERK signaling using dissociative inhibitors that target the interactions between signaling proteins. The dissociative inhibitors can disrupt specific steps of signal transduction which may restore a precise regulation of the uncontrolled signaling. For the screening of dissociative inhibitors of ERK pathway, we utilized the bimolecular fluorescence complementation (BiFC) assay to monitor the interactions. We targeted the assembly of KSR1 scaffold complex, which is necessary for the transduction for proper signaling. The N-terminal or C-terminal fragment of a fluorescent protein is fused to a pathway kinase or KSR1, respectively, and the fluorescence is restored when the selected proteins interact to reconstitute a functional fluorescent protein. The fluorescence intensity was monitored from cells co-expressing these fusion proteins to screen chemical libraries for dissociative inhibitors. After the serial elimination of false-positives, we identified two chemicals as candidates which seem to inhibit the interaction between the kinase MEK and the KSR scaffold. The dissociative inhibitors are postulated to reduce the possibility of system-wide shutdown or perturbation of signaling which is often occurred when targeting receptors or kinases using competitive inhibitors. Specific Inhibition of designated interactions using dissociative inhibitors allows for a powerful means to control or fix the misregulated signaling in many diseases caused by signaling disorders.

P498**Board Number: B645****Csk-Binding-Protein mediates Thy-1 signaling and neurite retraction caused by astrocyte $\alpha\beta 3$ integrin in neuronal cultures.**H. Maldonado^{1,2}, L. Leyton^{1,2,3}, A. Quest^{1,3}, R. Herrera-Molina⁴;

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Upon injury in the Central Nervous System, astrocytes migrate to the damaged area generating a non-permissive environment for neuronal regeneration. In general, this can be explained because glial cells possess in its cellular membrane or release outgrowth inhibitory molecules. Previously, we described that the astrocyte- $\alpha\beta 3$ Integrin binds to neuronal Thy-1 engaging pathway that involve Src inactivation leading to neurite retraction. However, how does Thy-1, a membrane GPI anchored protein, trigger intracellular signaling pathways is unknown. Csk Binding Protein (CBP) has been described as a Src-family scaffolding protein that precipitates with Thy-1 in other cellular lines. Thus, we propose the participation of this transmembrane-adaptor protein as a transducer of Thy-1 signaling to the cellular interior. Src inactivation can lead to an increase in ROCK activity, event that causes neurite retraction through actin cytoskeleton disruption by phosphorylation of Cofilin and Myosin light chain II (MLCII). Hypothesis: CBP and Thy 1 form a membrane complex that mediates Src inactivation, thus increasing phosphorylation of ROCK downstream effectors and neurite retraction caused by the addition of astrocyte $\alpha\beta 3$ integrin. Methodology: To study CBP participation we knock down CBP in neuron-like CAD cells. Recombinant $\alpha\beta 3$ integrin was used to stimulate transfected cells for 40 minutes and cells were then followed by phase-contrast microscopy to measure neurite length. We also evaluated MLCII, Cofilin and inactivating phosphorylation of Src (Y527) by Western blotting. To study CBP, Thy-1 and Src interactions we performed co immunoprecipitation and co localization assays using CAD cells and cortical rat neurons (E18, 14 days in vitro). Results: CBP knockdown prevented neurite retraction, the increase in inactivating phosphorylation of Src and the enhanced MLCII and Cofilin phosphorylation caused by $\alpha\beta 3$ integrin stimulation. CBP, Thy-1 and Src co precipitated in rat cortical neurons. The $\alpha\beta 3$ integrin addition enhanced co-localization, in the tip of neuronal processes, of CBP-Thy-1 and Thy-1-pSrcY527, as well as a decrease in co-localization of CBP with Src in both rat cortical neurons and CAD cells. Discussion: These results indicate that CBP and Thy-1 form a membrane complex that transduces $\alpha\beta 3$ Integrin-induced signals to the cell interior leading to Src inactivation and increase MLCII and Cofilin phosphorylation of thereby triggering neurite retraction. Finally, learning about Thy-1-associated signaling could be useful to identify therapeutic targets to assist neuronal regeneration. Supported by: FONDECYT 1110149(LL) and 1130250(AFGQ); BNI P09-015-F(LL) and Anillo ACT1111(AFGQ); Conicyt 24121665(HM), Becas Chile(HM).Conicyt-FODAP15130011 (AFGQ).

P499**Board Number: B646****Identifying a role for tetraspanin CD82 in cell cycle regulation of hematopoietic stem and progenitor cells.**C.A. Saito Reis¹, K.D. Marjon¹, K.L. Karlen¹, C.M. Termini¹, J.M. Gillette¹;¹Department of Pathology, University of New Mexico Health Sciences Center, Albuquerque, NM

Hematopoietic stem and progenitor cells (HSPCs) are specialized cells responsible for the continued production of new blood and immune cells. Cell signaling and adhesion within the bone marrow microenvironment tightly regulates the self-renewal and differentiation of HSPCs, which is critical for the function and maintenance of these cells. The tetraspanin, CD82, is a membrane scaffold protein shown to be highly expressed on HSPCs where it plays a role in modulating homing and engraftment to the bone marrow. Previous work in the lab showed that CD34+ cells in the G0 phase of the cell cycle were enriched in polarized CD82 domains critical for homing and engraftment to the bone marrow. Therefore, we hypothesize that CD82 expression and organization is a critical regulator of proliferation and cell cycle progression in HSPCs. To test this hypothesis we are utilizing the acute myelogenous leukemia progenitor-like cell line, KG1a, as a model for HSPCs. Taking a structure/function approach to evaluate the role of CD82, we generated CD82 knockdown (KD) (shRNA) and CD82 overexpressing (OE) cells (mCherry-CD82 vector). In addition, we generated a series of CD82 cell lines with mutations in N-glycosylation sites (NGLY: site directed mutagenesis Asp to Glu), palmitoylation sites (PALM: site directed mutagenesis Cys to Ser), and a C-terminal sort motif (YXX ϕ : amino acid change YSKV to ASKA). Next, we assessed the proliferation and cell cycle rate of these cell lines. Our data suggest that the CD82 PALM mutant and CD82 KD cells have increased proliferation when compared to control, OE, NGLY, and YXX ϕ cells. The proliferation rate of these cells did not change regardless of the presence of the ligand fibronectin or N-cadherin. In addition, we are utilizing the CD82 knock out (KO) mouse to evaluate changes in the HSPC population, which include the quantity of HSPCs, their localization, and also their self-renewal and differentiation potential. Preliminary data from these experiments indicate that the loss of CD82 expression does alter the HSPC population and self-renewal potential is likely to play a specific role. In combination, these data implicate CD82 expression and its membrane organization as a critical regulator of the cell signaling that occurs between HSPCs and the bone marrow microenvironment, which in turn can modulate the self-renewal potential of HSPCs.

P500**Board Number: B647****FRET biosensors reveal Ca²⁺/PKA crosstalk through AKAP targeting dynamics.**M.B. Schott¹, F.N. Thompson Gonowolo¹, B.D. Grove¹;¹Basic Sciences, University of North Dakota, Grand Forks, ND

A-Kinase Anchoring Proteins (AKAPs) play a critical role in cellular homeostasis by scaffolding cAMP-dependent Protein Kinase A (PKA) and other signaling enzymes in proximity to downstream effectors.

Although they are named “anchoring proteins”, some AKAPs do not assemble static enzyme complexes but form dynamic signalosomes that traffic to different subcellular compartments in response to stimuli. Gravin (AKAP12), a multivalent scaffold linked to a variety of cellular functions, anchors PKA and other enzymes to the plasma membrane but is known to undergo redistribution to the cytosol upon intracellular Ca^{2+} elevation. We postulate that gravin redistribution represents a novel crosstalk mechanism between Ca^{2+} -dependent and cAMP-dependent signaling pathways. To assess this, we measured the impact of gravin-V5/His expression on compartmentalized PKA activity using the PKA FRET biosensor AKAR3. In cultured AN3 CA cells which lack endogenous gravin, expression of gravin-V5/His caused an elevation in forskolin-stimulated PKA activity in AKAR3 constructs targeted to the plasma membrane compared to control cells lacking gravin or expressing a Δ PKA-gravin construct missing the PKA-binding domain. In contrast, gravin-V5/His expression caused a decrease in forskolin-stimulated PKA activity in cytosolic AKAR3 constructs compared to control cells lacking gravin or expressing Δ PKA-gravin. Interestingly, gravin localization at the cell periphery was dramatically impaired by the mutation of a calmodulin-binding consensus sequence within gravin’s CB4 domain. Similar to control cells, this mutant gravin construct had no effect on membrane and cytosolic PKA activity. Finally, pretreatment with the calcium-elevating agent thapsigargin caused the redistribution of gravin away from the plasma membrane and prevented gravin-mediated elevation in plasma membrane PKA activity in response to forskolin. These results reveal that gravin shapes the subcellular profile of PKA activity and support the hypothesis that gravin mediates crosstalk between Ca^{2+} and cAMP-dependent signaling pathways. Based on these results, AKAP localization dynamics may represent an important paradigm for the regulation of cellular signaling networks.

P501

Board Number: B648

Phase-transitions of Multivalent Adapter Proteins.

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Heterogeneity of the plasma membrane in the form of clustering of proteins and lipids is well documented in many signaling pathways. However, the general mechanisms behind this organization remain unclear. Here, we report the in-vitro reconstitution of membrane clusters, using the adhesion protein Nephrin and its cytoplasmic partners Nck and N-WASP. We quantitatively determine that the clustering occurs due to a two-dimensional phase-separation process, induced by polymerization of the proteins through multivalent interactions. The clusters are sites of actin polymerization, supporting the hypothesis that clustering of nucleation promoting factors could be a general way to localize actin assembly on the plasma membrane. Furthermore, we show that an unstructured region of the adapter protein Nck self-assembles, and in cooperation with the multivalent interactions of the structured regions, induces phase-separation. Therefore, unstructured regions in adapter proteins may be specifically designed to provide additional energy for clustering of multivalent signaling proteins. Our

studies demonstrate that protein-protein interactions are sufficient to produce clusters on the membrane through phase-transition, and that cytoplasmic adaptor proteins can act as ligands to organize membrane receptors into signaling zones.

P502

Board Number: B649

Megadalton nodes at cell cortex link cell growth and division.

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Cell cycle progression is coupled to cell growth, but the mechanisms that generate growth-dependent cell cycle progression remain unclear. Fission yeast cells enter into mitosis at a defined size due to the conserved mitotic inducer Cdr1, which inhibits Wee1 and localizes to a set of cortical nodes in the cell middle. Through a large-scale protein interaction screen, we found that the conserved cell polarity protein Skb1 binds to Cdr1 and its inhibitory target Wee1. Skb1 inhibited mitotic entry through negative regulation of Cdr1 but localized to a novel set of previously unidentified nodes at the cell cortex. Through live-cell imaging, we demonstrated that Cdr1 nodes and Skb1 nodes are distinct structures at the plasma membrane, and artificial targeting experiments confirmed that spatial separation of these distinct node structures is required for proper entry into mitosis. Thus, Skb1 nodes operate as spatial cues for the signaling pathways that link cell size with mitotic entry. To understand the mechanisms of Skb1 node formation, we screened for interaction partners and identified the uncharacterized protein Slf1 as an Skb1 ligand. Skb1 and Slf1 colocalized in cortical nodes, and were mutually dependent for node assembly. Using quantitative fluorescence microscopy and in vitro assays, we discovered that Skb1-Slf1 nodes are megadalton structures that are anchored to the membrane by a lipid-binding region in the Slf1 C-terminus. Skb1 and Slf1 are present at a 1:1 molar ratio in nodes, and genetic epistasis experiments showed that these two proteins function in a linear pathway to control mitotic entry through Cdr1 and Wee1. Our combined experiments have identified a novel cortical compartment that assembles through defined molecular interactions. Further, the formation of distinct microdomains at the plasma membrane couples cell size to mitotic entry through spatial control of signaling pathways.

P503**Board Number: B650****Binding of different SH3 domain-containing proteins to the ubiquitin ligase Itch proline-rich region.**G. Desrochers¹, L. Cappadocia², M. Lussier-Price², J.G. Omichinski², A. Angers¹;¹Sciences Biologiques, Université de Montréal, Montréal, QC, ²Biochimie, Université de Montréal, Montréal, QC

Itch is a member of the C2-WW-HECT (CWH) family of ubiquitin ligases that also includes Nedd4 in humans and Rsp5p in yeasts. In addition to the WW substrate recognition domains common to all members of the CWH family, Itch contains a conserved proline-rich region (PRR) that enables it to interact with SH3 domain-containing proteins. The PRR of Itch is composed of 20 amino acids and contains four consensus class I and class II SH3-binding motifs. Although SH3 domain-containing partners of Itch have been identified, there is very little known about their binding preferences towards Itch.

In this work, we compare the binding of Endophilin, β -PIX, Pacsin, and Amphiphysin using isothermal titration calorimetry (ITC). We measured the dissociation constants of Itch's PRR for the isolated SH3 domain from the four proteins of interest. Based on these experiments, there is striking difference in the affinity of the isolated SH3s for Itch, with K_d 's varying over a thousand fold. In all cases, the binding stoichiometry is 1:1 except for the SH3 domain of β -PIX, which is 2:1 as previously reported.

In addition, we generated GST-fusion proteins with overlapping sections of the PRR of Itch and performed pull-down assays to determine if the SH3 domain-containing proteins display distinct binding preferences. We found that these proteins bind different yet overlapping motifs in the N-terminal region of the PRR, whereas β -PIX makes extensive contact with the central region of the Itch PRR. To further refine these findings, we generated point mutations at each of the five Arg residues within the PRR motif. GST pull-down assays using these mutants showed that the first three Arg residues all contribute to various extents to binding to the different SH3 domains.

Our findings show that although these proteins bind overlapping sequences, they display distinct preferences and affinities. Interestingly, these SH3 domain-containing partners are all readily ubiquitylated by Itch in cellular assays, except for β -PIX. Thus, in addition to their binding properties, there are important functional differences for their interaction with Itch. Taken together, these results expand our comprehension of the specific roles of Itch, and provide further knowledge of the interplay between protein ubiquitylation, cell signalling and protein trafficking.

P504**Board Number: B651****A physical explanation for protein partitioning in lipid membranes.**C.B. Carbone¹, X. Su¹, E. Hui¹, R.D. Vale^{1,2};¹Department of Cell and Molecular Pharmacology, University of California, San Francisco, San Francisco, CA, ²The Howard Hughes Medical Institute, San Francisco, CA

Receptor-ligand binding across two plasma membranes is a recurrent phenomenon in cell biology. It has been theorized that the lipid membranes may be important in controlling how receptors and other proteins organize at the membrane interface. Specifically, the 'kinetic segregation' model, posits that proteins with large extracellular domains might be excluded from regions of close membrane apposition, and that receptor ligand pairs may organize based on intermembrane distance. Our recent development of an *in vitro* system using giant unilamellar vesicles (GUVs) and supported lipid bilayers (SLBs) has allowed us to test the effect of intermembrane distance on protein partitioning using a combination of timelapse, single molecule, and interference microscopy. Our ability to examine this biophysical phenomenon *in vitro* may provide important insights into how proteins organize in the immunological synapse.

P505**Board Number: B652****PLASMA MEMBRANE ORGANIZATION PROMOTES CD36 SIGNAL TRANSDUCTION IN ENDOTHELIAL CELLS.**J.M. Githaka¹, A. Vega², M.W. Davidson³, K. Jaqaman², N. Touret¹;¹Department of Biochemistry, University of Alberta, Edmonton, AB, ²Department of Biophysics, University of Texas Southwestern Medical Center, Dallas, TX, ³National High Magnetic Field Laboratory and Department of Biological Science, Florida State University, Tallahassee, FL

CD36 is a multiligand scavenger receptor that ligates Thrombospondin-1 (TSP-1) at the surface of endothelial cells and induces their apoptosis. Recent evidences have shown that clustering of CD36 is necessary for signal transduction in macrophages and is regulated by the architecture of the cortical actin cytoskeleton apposed to the plasmalemma. Here, we investigated the role of the cortical actin cytoskeleton and plasma membrane nanodomains in the control of CD36 activation in endothelial cells. Stimulation with multivalent ligands (TSP-1 and anti-CD36 IgM) resulted in the downstream phosphorylation of the Src Family kinase, Fyn. Disruption of the actin cytoskeleton or removal of cholesterol blocked this activation. To gain molecular details on the rearrangement of the receptors during TSP-1 binding, we conducted superresolution approaches (based on PhotoActivated Localization Microscopy or PALM) and quantitative spatial distribution analysis. Endothelial cell lines stably expressing CD36-PAmCherry were generated for that purpose. At steady state, CD36 receptors pre-exist in small clusters (average diameter of 100 nm), in which Fyn was also present. Upon TSP-1 binding, CD36 clusters increased in size (average diameter of 140 nm) and also became denser. The average distance

between CD36 molecules in these clusters was in the range of 8 nm compare to 11 nm in the control condition. F-actin depolymerization or cholesterol depletion reduced the capacity of the ligand to induce formation of larger clusters resulting in a decreased recruitment and activation of Fyn. Our data demonstrate cooperation between cholesterol-dependent domains and the cortical actin cytoskeleton in the organization of CD36 receptors and Fyn before and during TSP-1 stimulation.

Post-translational Modifications in Signaling

P506

Board Number: B653

Maspin is phosphorylated in MCF-10A cells.

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Maspin (mammary serpin) is a 42 kDa tumor suppressor protein which belongs to superfamily of serpins due to sequence homology, however, its mechanism of action does not depend on protease inhibition. This protein was identified in 1994 during a search for genes expressed in normal mammary epithelial cells but absent in breast tumors. Among maspin biological effects are modulation of cell adhesion, inhibition of tumor growth, invasion and angiogenesis, a pro-apoptotic effect and control of oxidative stress response. This functional diversity reflects maspin numerous ligands and its subcellular localization, since it is found on the plasma membrane, in the cytoplasm, nucleus and in mitochondria. Several studies indicate that maspin is regulated by posttranslational modification, yet, little is known about how these alterations regulate its biological activities, subcellular localization and the intracellular pathways involved. Using the non-transformed mammary epithelial cell line MCF-10A as a model system, we identified four different maspin forms by 2D-SDS-PAGE followed by immunoblot with anti-maspin. Three of these forms were sensitive to acidic phosphatase treatment, suggesting that they are phosphorylated. Sodium peroxidovanadate treatment, a potent tyrosine phosphatase inhibitor, resulted in a rapid increase in maspin protein levels as well as its cytoplasmic accumulation. These data suggest that phosphorylation may be involved in maspin subcellular localization and regulation of its levels in the cell. Interestingly, we found that maspin is highly phosphorylated in a metastatic cell line which exhibits maspin predominantly in the cytoplasm. Thus, we verified a correlation between tyrosine phosphorylation, cytoplasmic localization and tumor progression. Searching for signaling pathways involved in maspin phosphorylation, we found that EGF-treatment increases maspin phosphorylation. This observation suggests that maspin may take part of the EGF signaling pathway. An association between EGF signaling, maspin phosphorylation and its subcellular localization is currently under investigation. Altogether, these results shed new light into the molecular mechanism underlying maspin biological activities and how this mechanism might be altered in tumor cells. This research was supported by CNPq and Fapesp.

P507**Board Number: B654****Possible role of UDP-GlcNAc 2-epimerase /ManNAc kinase (GNE) in cell apoptosis.**R. Singh¹, R. Arya¹;¹School of Biotechnology, Jawaharlal Nehru University, New Delhi, India

Sialic acids are nine carbon acidic amino sugars present at terminal end of monosaccharides on most mammalian glycoconjugates. Correct sialylation of glycoproteins is essential for normal cell function as they play crucial role in various biological, pathological and immunological processes. Genetically determined abnormalities of the glycosylation processes have been identified as a cause of severe skeletal muscle/neuromuscular disorders such as Hereditary Inclusion Body Myopathy (HIBM)/ Distal myopathy with rimmed vacuoles (DMRV). Still biological basis of pathogenesis is not known. Sialic acid synthesis is regulated by a rate limiting bifunctional enzyme, UDP-GlcNAc 2-epimerase /ManNAc kinase (GNE) that possess N-terminal epimerase and C-terminal Kinase domain. Homozygous missense mutations in either epimerase or kinase domain of GNE results in hyposialylation of glycoproteins in muscle cells of patients and primary defect in either N or O-linked glycosylation. However, it appears from some recent experiments including those from our laboratory that mutant GNE may also affect targets that are not directly related to sialic acid biosynthesis. In particular cytoskeletal network, sarcomere organization and apoptotic signaling are likely to be altered in muscle cells. In the present study, we have used HEK cell based model system for HIBM already established in the laboratory to understand cell apoptosis in presence or absence of functional GNE. We have used wild type GNE and pathologically relevant GNE mutations alongwith GNE knockdown stable HEK cell lines to study cell proliferation and apoptosis. We observed significant reduction in cell proliferation of GNE mutant and knockdown cells compared to vector control. Further staining with Annexin V-FITC and Tunel assay depicted significant increase in number of apoptotic cells and nuclear fragmentation of GNE mutant cells. In addition, changes in the structure and function of mitochondria were observed in GNE deficient cell lines. Our results clearly indicate that mutation in GNE affects cellular apoptosis and may contribute to the pathogenesis of the disease.

P508**Board Number: B655****Tubulin tyrosine ligase-like 4 is important for maintaining the structural integrity of erythrocyte plasma membrane.**F. Ijaz¹, K. Tsutsumi², Y. Hatanaka³, T. Hatanaka³, S. Asai-Akeida³, N. Zaima^{1,3}, K. Ikegami¹, M. Setou¹;¹Department of Cell Biology and Anatomy, Hamamatsu University School of Medicine, Hamamatsu, Japan, ²Division of Cell Biology, Department of Biosciences, School of Science, Kitasato University, Kanagawa, Japan, ³Mitsubishi Kagaku Institute of Life Sciences (MITILS), Tokyo, Japan

Glutamylation is a kind of post-translational modification in which multiple glutamic acids are added to gene-encoded glutamic acid residues in target proteins. Glutamylation occurs on tubulin and some non-tubulin proteins in various types of cells. This modification is catalyzed by some members of tubulin tyrosine ligases-like (TTL) protein family. TTL4 is a glutamylation-initiating glutamic acid ligase (E-ligase), and is known to glutamylate several non-tubulin proteins. In this study, we demonstrate the important role of TTL4 in red blood cells (RBCs) independent of tubulin glutamylation. We found nucleosome assembly protein 1 (NAP1) to be the sole protein in RBCs that was detected by a glutamylation-specific antibody, GT335. The glutamylated NAP1 was detected more strongly in plasma membrane than in cytoplasm of RBCs. The glutamylation of NAP1 completely disappeared in RBCs of TTL4-deficient mice. Hematological analysis showed larger diameters of RBCs in TTL4-deficient mice. Furthermore, the knockout mice showed increased hemolysis in mouse model of acute hemolytic anemia. Electron microscopy of RBC plasma membrane revealed significant decrease in spectrin-actin lattices in TTL4-deficient samples compared to wild-type samples. Taken together, our data suggest that the TTL4-mediated glutamylation of NAP1 is involved in maintaining RBC membrane structures.

P509**Board Number: B656****Poly(ADP-ribose) polymerase-1 (PARP-1) activity is regulated by a domain that locally unfolds when PARP-1 binds DNA damage.**J.M. Dawicki-McKenna¹, M.F. Langelier², J.E. DeNizio¹, A.A. Riccio², C.D. Cao¹, K.R. Karch¹, M. McCauley², B.E. Black¹, J.M. Pascal³;¹Department of Biochemistry and Biophysics, University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA, ²Department of Biochemistry and Molecular Biology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA, ³Department of Biochemistry and Molecular Pharmacology, Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA

PARP-1 plays an important role in the cellular response to DNA damage. It rapidly localizes to DNA breaks where it couples damage detection to poly(ADP-ribosyl)ation (PARylation) of target proteins. The covalent attachment of PAR, primarily to PARP-1 itself, initiates the recruitment of several DNA repair factors to the sites of damage. Binding to DNA damage allosterically activates PARP-1 catalytic activity.

Despite valuable insight provided by the crystal structure of PARP-1 in complex with DNA damage (PDB ID 4dqy), the details of how DNA binding is coupled to increased PAR production that takes place more than 45 Å away in the active site remain unclear. Complementary approaches are now required to relate information provided by static structures to dynamic changes that occur in solution when PARP-1 is active. Here, we use hydrogen-deuterium exchange coupled to mass spectrometry (HXMS) to report differences in the structure and dynamics of full-length PARP-1 bound and unbound to a DNA single-strand break (PARP-1/DNA). Our HXMS results unexpectedly reveal a regulatory domain of PARP-1 that locally unfolds when PARP-1 is activated by DNA damage. The regulatory domain in PARP-1 is well folded prior to encountering DNA, but contains a large region that unfolds when PARP-1 recognizes DNA damage. We designed and tested mutants that revealed how allosteric activation of PARP-1 works. Unfolding of the regulatory domain is prevented in a PARP-1 mutant that cannot be activated by DNA binding. Further, we tested a mutant suspected of destabilizing the folding of the regulatory domain, and found that it is destabilized relative to the wild type protein and also has a higher basal activity. Using our HXMS data and existing crystal structures, we designed a mutation to disconnect the allosteric communication resulting from binding to damaged DNA. Indeed, this mutant no longer responds enzymatically to DNA damage. Taken together, our data support a simple and elegant model for connecting local protein unfolding to the allosteric activation of a major eukaryotic DNA repair enzyme. Our model provides a sophisticated view of the molecule in solution that is likely to be useful when targeting PARP-1 with small molecules, as is currently being done for several clinical trials.

P510

Board Number: B657

Analysis of molecular mechanism for Gas ubiquitination.

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G proteins consist of α , β and γ subunits and undergo an activation-inactivation cycle depending on the binding of guanine nucleotides. They function as molecular switches that turn on intracellular signaling cascades in response to the activation of G protein-coupled receptors (GPCRs) by extracellular stimuli including hormones, light and odorous chemicals. We have recently reported that Gas is degraded by the ubiquitin-proteasome pathway and its associated protein, Ric-8B, suppresses ubiquitination of Gas. These data raise a possibility that Gas is quantitatively controlled by ubiquitin-dependent degradation and Ric-8B stabilizes Gas by antagonizing ubiquitination. However, molecular mechanisms underlying ubiquitination of Gas are still unclear. We performed proteomic screening to identify Gas- or Ric-8B-binding partners that are involved in Gas ubiquitination. Flag-tagged Gas or Ric-8B were expressed in HEK293T cells and we analyzed by tandem mass spectrometry following immunoaffinity chromatography. Candidate proteins including one E3 ubiquitin ligases, GsAP (Gas-associating protein) - 1 and three deubiquitinases, GsAP-2, GsAP-3 and GsAP-4 were identified. We observed that ectopically expressed Flag-tagged GsAP-1, -3, or -4 and Gas in HEK293T cells were coprecipitated. To investigate

whether candidate proteins affect G α s ubiquitination, we expressed these proteins and G α s with His-tagged Ubiquitin in HEK293T cells, and performed pull-down assay using Ni-NTA agarose. We found that GsAP-3 suppressed G α s ubiquitination, but not GsAP-4. Knockdown experiments using siRNAs are currently ongoing and in this presentation we will discuss possible molecular mechanisms of G α s ubiquitination.

P511

Board Number: B658

Ubiquitination and the control of Plasmodium falciparum cell cycle by melatonin.

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The ubiquitin proteasome system (UPS) has several roles in eukaryotes including cell-cycle control, transcription regulation and signal transduction. Post-translational modifications such as ubiquitination are important modulator of signaling processes, cell cycle and many other cell functions. There are increasing evidences that melatonin and ubiquitin/proteasome system (UPS) interacts to regulate many cellular functions. We previously reported that melatonin-induced up-regulation of gene expression related to UPS in Plasmodium falciparum. Interestingly, the response to melatonin treatment is completely abolished in Pfk7 knockout of the parasites, and the response is restored when the parasites are complemented with Pfk7 gene, suggesting a link between modulation of intraerythrocytic differentiation of the parasite and this hormone. In this work we demonstrate by qPCR analysis, that melatonin induces gene up-regulation of nine out of fourteen genes from UPS. The induction of UPS gene expression occurs between 4 to 5 hours after hormone treatment, which leads to a time-dependent differential protein ubiquitination in parasites. We also performed deep sequencing (RNA-seq) polyA + extracted from trophozoites of Plasmodium falciparum (3D7 and Pfk7 knockout strains) treated with melatonin to identify possible targets and evaluate the role of protein 7 kinase (PK7). The results revealed that melatonin causes a temporally controlled gene expression of UPS members followed by a differential protein ubiquitination modulation. Finally, we identified two possible candidates for ubiquitination in response to melatonin: S-adenosylmethionine synthase and eIF4A-like protein. Importantly, the former is involved in cell growth and apoptosis in mammals and in developmental regulation in plants while the later is involved in P. falciparum proliferation suggesting that such substrates could be modulators of the parasite cell cycle and regulated by melatonin signaling.

P512**Board Number: B659****A 19S proteasomal subunit-PSMD9 enhances I κ B α degradation and NF- κ B activation by interacting with hnRNPA1.**I. Sahu¹, N. Sangith¹, M. Ramteke¹, R. Gadre¹, P. Venkatraman¹;¹Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Navi Mumbai, India

Background: PSMD9 is a PDZ-domain containing non-ATPase subunit of the 26S proteasome. Besides its well-studied role in proteasome assembly, the structure and functions of PSMD9 are largely uncharacterized. Till date a large number of protein-protein interactions have been identified by various proteomic methods and yet only a little of them turn out to be biologically significant. Using a novel structural bioinformatics method and peptide based screening we recently reported that, heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1) is one among the novel interacting partners of PSMD9.

Results: We demonstrated in HEK293 and HeLa cells that, PSMD9 through its PDZ-domain interacts with the C-terminal motif of hnRNPA1. This domain-motif interaction is responsible for proteasomal degradation of I κ B α and consequently influences the NF- κ B activation. In accordance, overexpression of PSMD9 enhances both the basal and TNF- α induced I κ B α degradation as well as NF- κ B activation (upto 2-4 fold) while knock down of PSMD9 results in the opposite effect. Point mutations (Q181G and a triple mutation- L124G/Q126G/E128G) in PDZ domain of PSMD9 or 7-amino acid deletion from C-terminus of hnRNPA1 disrupt interaction between the two proteins. Consequently, overexpression of these mutants has no effect on either basal or TNF- α induced I κ B α degradation and NF- κ B activation. In addition, overexpression of WT-hnRNPA1 fails to modulate NF- κ B activation in PSMD9 knockdown background. These results emphasize the importance of interaction between PSMD9 and hnRNPA1 for this cellular function. In vitro interaction studies suggest hnRNPA1 interacts with I κ B α directly, whereas PSMD9 interacts only through hnRNPA1. Furthermore, hnRNPA1 shows increased association with the 26S proteasome upon PSMD9 overexpression and/or TNF- α treatment which however has no such effect in the absence of PSMD9. Endogenous and trans-expressed PSMD9 are found associated with the proteasome complex and this association is unaffected by PDZ-mutations or TNF- α treatment. Moreover, neither overexpression of the WT or mutant PSMD9 and hnRNPA1 nor the down-regulation of PSMD9 had significant effect on the Proteasomal activity in HEK293 cells as such.

Conclusion: Collectively, the tripartite interactions between I κ B α , hnRNPA1 and proteasome bound PSMD9 illustrates a potential mechanism by which ubiquitinated I κ B α are recruited on proteasome for degradation. In this process, hnRNPA1 may act as a shuttle receptor and PSMD9 as a subunit acceptor. The PSMD9 and hnRNPA1 interface/hot spot site may emerge as a vulnerable drug target in cancer cells which require consistent NF- κ B activity for survival.

P513**Board Number: B660****Arginylation affects G-protein signaling and visual processing in the retina.**D.W. Dong^{1,2}, M.E. Fina¹, S.S. Nikonov³, N. Vardi³, J. Wang¹, A.S. Kashina¹;¹Department of Animal Biology School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, ²Penn Genome Frontiers Institute, University of Pennsylvania, Philadelphia, PA, ³Department of Neuroscience School of Medicine, University of Pennsylvania, Philadelphia, PA

Arginylation is a posttranslational modification that has been recently implicated in multiple key processes in development and physiology. Here we studied the effect of the neuron-specific knockout of arginyltransferase (Ate1) on visual processing in the retina using a conditional knockout model with Ate1 deletion driven by brain-specific Nestin (Nes) promoter. Electroretinography studies showed a thirty percent increase of the response of the dark-adapted retina bipolar neurons to dim light flashes in Nes-Ate1 mice compared to age-matched controls. Immunohistochemistry of retina sections showed an increase in one of the regulators of G-protein signaling (RGS) in Ate1 knockout, RGS11, likely due to inhibition of its arginylation-dependent degradation by the N-end rule pathway. Since the dim-light responses in rod bipolar neurons are facilitated by RGSs accelerating G-proteins' GTP hydrolysis rates, increased RGS protein levels leads to increased dim-light responses. Our data suggest that Ate1-mediated arginylation affects G-protein signaling in the retina by regulating RGS proteins, facilitating the proper balance of RGS family members in the healthy retina, and ensuring normal processing of visual stimuli in the healthy animals.

P514**Board Number: B661****Arginylation Regulates Purine Biosynthesis by Facilitating the Biological Activity of Phosphoribosyl Pyrophosphate Synthase.**F. Zhang¹, K. Colavita², I. Rodionova³, B. Buckley⁴, D. Scott⁵, A. Kumar¹, S. Shabalina⁶, S. Saha⁷, M. Chernov⁸, A. Osterman⁵, A.S. Kashina⁹;¹School of Medicine, University of Miami, Miami, FL, ²University of Pennsylvania, Philadelphia, PA, ³Sanford Burnham Medical Research Institute, LaJolla, CA, ⁴Roswell Park Cancer Research Center, Buffalo, NY, ⁵Sanford Burnham Medical Research Institute, LaJolla, CA, ⁶NCBI, National Institutes of Health, Bethesda, MD, ⁷Tezpur University, Napaam, India, ⁸Roswell Park Cancer Institute, Buffalo, NY, ⁹Department of Animal Biology School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA

Protein arginylation is an emerging posttranslational modification that targets a number of metabolic enzymes, however the mechanisms and downstream effects of this targeting are unknown. Here we investigated arginylation of PRPS2, the key enzyme of purine biosynthesis, previously found in our proteomic screen to be arginylated at the N-terminus, unlike its close homolog PRPS1. We found that such selective arginylation of PRPS2 is regulated through a coding sequence-dependent mechanism that

combines elements of mRNA secondary structure with Lys residues encoded near the N-terminus in PRPS1 but not PRPS2. Lack of arginylation renders cells vulnerable to purine nucleotide synthesis inhibitors and affects related Gly and Ser metabolic pathways. Measurements of PRPS2 activity in cell extracts show that arginylation greatly facilitates the biological activity of this enzyme. This study is the first demonstration of a direct effect of arginylation on enzymatic activity, and the first implication of arginylation in nucleotide metabolism.

P515

Board Number: B662

Identifying calpain-2 interacting proteins in endothelial cell lysates through affinity purification coupled with mass spectrometry.

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Calpain-2, a ubiquitous, calcium dependent proteolytic enzyme, is implicated in a variety of endothelial cell (EC) functions and responses including signal pathways downstream of flow/shear stress, VEGF, angiotensin II, and integrins. How calpain's presence, or activity, results in the measured responses/outputs is not yet described. An affinity capture-mass spectrometry strategy to screen for the proteins that bind to calpain-2 was utilized in order to begin to identify the individual protein substrates that calpain modifies, degrades or targets in ECs. This strategy required use of a catalytically incompetent variant of calpain-2 (C105A) that retains its native conformation and is also fused to a C-terminal peptide substrate for the biotin protein ligase BirA. The purified, biotinylated C105A-calpain-2 served as 'bait' and soluble extracts of bovine aortic endothelial cells (BAEC) were used as 'prey' after first removing endogenously biotinylated proteins and inactivation of endogenous calpains by reaction with a covalent inhibitor (suc-LLY-CHN2). Identifying proteins bound to calpain in the presence of calcium offered two key advantages: 1) the calpain-2 bait remained bound to the resin after EGTA elution of interacting proteins and 2) the capture of the well characterized calpain inhibitor, calpastatin, and/or other known putative substrates would provide proof of concept for the experimental approach. Calpain-protein complexes were captured with streptavidin-ultralink resin (Thermo-Pierce), washed with a series of buffers and proteins of interest recovered by elution with EGTA/EDTA. Proteins eluted from the biotinylated C105A-calpain-2 were compared with proteins isolated in a parallel control reaction containing non-biotinylated C105A-calpain-2 and free biotin. Of approximately 30 proteins initially identified by LC-MS-MS using Protein-Pilot™ software about 25% were also present in the control sample. Nonspecifically bound proteins were highly abundant in the lysates and included GAPDH, HSPs (D1,A9, A5,B1) and fetuin (AHSG). Not surprisingly calpastatin, calpains' nanomolar affinity inhibitor was the highest scoring calpain interacting protein. The selective capture of several annexins including ANXA1, ANXA2, ANXA6 and ANXA4 was of particular interest. ANXA1 was chosen to confirm calpain's ability to bind to it and we successfully demonstrated calpain-2 cleavage of it. Extension of these studies

and further analysis of proteins captured will allow design of future experiments aimed at defining calpain's mechanistic links to specific physiological and/or pathological events in ECs as well as other cell types

P516

Board Number: B663

Identifying Wntless interaction domains required for its function in Wnt signaling.

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Wnt signal transduction plays important roles in numerous phases of organismal development and tissue homeostasis. When aberrant, can drive cell transformation that results in cancer and various developmental diseases. Over the years, it has become clear that Wnt activity is strictly regulated, not only in signal receiving cells, but also in signal producing cells at the level of Wnt production, transport and secretion. Posttranslational modifications of Wnts are critical for Wnt signal regulation at the level of intracellular trafficking, extracellular dissemination, and ligand-receptor interaction. Modified Wnts are escorted through the secretory pathway for extracellular release by Wntless (Wls), the dedicated Wnt chaperone protein. Wls directly interacts with lipid modified Wg (Drosophila Wnt) within the ER of the signal producing cells in order to assist its navigation through the secretory pathway for cell surface deployment, an indispensable step for downstream signal activation. Previous studies in our laboratory has shown that DWls (Drosophila Wls) forms homo-oligomeric structures required for Wg interaction, in which both its first transmembrane domain and amino acids between 137 to 223 play a role. While the presence of Wg binding region was not a requirement for DWls oligomers to form, DWls oligomerization was requisite for the Wg-DWls interaction; thus likely necessary for proper Wg intracellular transport. Recently, mutational studies in our lab revealed that cysteines, C50 and C72, within DWls are important for intermolecular disulfide bridges and oligomer formation. The effect of mutation of these cysteines in DWls-DWls and DWls-Wg interactions and overall DWls function remains to be determined.

P517

Board Number: B664

Control of Nrf2 signaling by O-GlcNAc.

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O-GlcNAcylation is the addition and removal of β -D-N-acetylglucosamine (GlcNAc) to serine and threonine residues of nuclear and cytoplasmic proteins. Similar to phosphorylation, with which it often competes, O-GlcNAcylation is reversible. This dynamic cycling is managed by the enzymes O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) to control a multitude of cellular processes, including

transcription, nutrient sensing, and cell cycle progression. Despite being essential and previously implicated in several diseases including cancer, major aspects of O-GlcNAc regulation are still poorly defined. Here, we used DNA microarrays and global gene expression analysis to evaluate the transcriptional response to 5SGlcNAc, a small molecule inhibitor of OGT. We found that 5SGlcNAc caused marked upregulation of several canonical targets of the transcription factor nuclear factor erythroid 2-related factor (Nrf2), a crucial regulator of redox homeostasis and cell survival responses to endogenous and exogenous stresses. Microarray data were confirmed by real-time PCR for Nrf2 target genes glutamate-cysteine ligase, modifier subunit (GCLM), NADPH dehydrogenase, quinone 1 (NQO1), and heme oxygenase 1 (HO1). Further, Nrf2 and HO1 protein expression increased for 5SGlcNAc treated samples with effects similar to that of positive control tert-Butylhydroquinone (tBHQ), a known inducer of Nrf2. We propose that O-GlcNAc represses Nrf2 signaling under basal conditions by enabling proteasome-mediated degradation of Nrf2 protein, linking intracellular glycosylation to redox homeostasis and stress responses. Experiments are underway to identify relevant substrate(s) and underlying mechanism through which O-GlcNAcylation suppresses Nrf2.

P518

Board Number: B665

RNF144A requires its transmembrane domain for E3 ubiquitin ligase activity.

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RNF144A E3 ubiquitin ligase can ubiquitinate DNA-PKcs and promote DNA damage-mediated apoptosis (Ho et al, PNAS, 2014). It also regulates breast cancer cell migration and invasion. Here we characterize an important regulation of RNF144A through its transmembrane domain. The transmembrane domain of RNF144A is highly conserved among species. Deletion of the transmembrane domain abolishes its membrane localization and also significantly reduces its E3 ubiquitin ligase activity. Further evidence shows that the transmembrane domain is required for RNF144A self-association and may partially through a classic GXXXG interaction motif. A membrane localization loss mutant of RNF144A still keeps its self-association and E3 ligase activity, suggesting these functions are independent of RNF144A membrane localization. Therefore, our data demonstrate that the transmembrane domain of RNF144A has at least two independent roles, membrane localization and activation of its E3 ligase activity.

Mechanotransduction 1

P519

Board Number: B667

Micro-fabricated devices with stretchable silicone substrates and micro-patterned silicone gels for experiments on adherent cells with high-resolution imaging.

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Flexible membrane substrates made of transparent silicone elastomers and experimental setups stretching those membranes unidirectionally or uniformly have been used for various experiments on different adherent mammalian cells. Stretching of the substrates, which can be one-time or periodic, can cause immediate and long-term mechanosensing cellular responses. However, in many of those experimental setups, cells on the flexible substrates cannot be accessed with short working distance (WD) high NA objectives, precluding high-resolution imaging of the cells in real-time. Moreover, when significantly stretched, large diameter membranes tend to move far out of focus, thus greatly complicating high-resolution imaging, even when the adherent cells are optically accessible. To address these problems, we have designed, built, and tested micro-fabricated devices, in which an ~100 μm thick, silicone membrane forms the bottom of an ~1mm wide micro-well. The membrane is stretched by the application of vacuum to two cavities flanking the micro-well. The design of the devices is optimized to limit the out-of-focus movement of the membrane to 5-10 μm , at lateral extensions as large as 12%, thus facilitating real-time high-resolution imaging. Using short WD, high NA oil-immersion and water immersion objectives and test fluorescent microparticles attached to the membranes, we demonstrated resolutions of ~0.3 μm . In a modified version of the device, with a high refractive index ($n = 1.46$) silicone membrane, 10% stretching was combined with total internal reflection fluorescence (TIRF) imaging. The setup has been used to stretch adherent neurons to study the effect of strain on axonal transport. In a separate project, we used micro-contact printing to generate micro-patterns of extracellular matrix (ECM) proteins on soft silicone gels with a high refractive index compatible with TIRF microscopy. To this end, we coated microscope cover glasses with 10-30 μm layers of TIRF-compatible gels and made microfabricated stamps with imbedded permanent magnets. The micro-patterned stamps were brought in a soft contact with the gel surfaces by activating an electromagnet, while monitoring the gel surfaces under a microscope. We used this technique to generate micro-patterns with ~8 μm wide strips on TIRF-compatible silicone gels with elastic moduli as low as 20 kPa. In a test experiment, we observed fibroblasts to exclusively attach to the ECM-patterned strips and to migrate along the strips for as long as 3 days.

P520**Board Number: B668****N-WASP/WIP mediated matrix adhesion site maturation drives 3D cancer cell migration through direct force coupling to the nucleus.**T. Zech¹, O. Chatzidoukaki^{1,2}, A. Charles-Orszag², S. Sender², L. Machesky²;¹Institute of Translational Biology, Dept of Cellular and Molecular Physiology, University of Liverpool, Liverpool, England, ²CRUK Beatson Inst Cancer Res, Glasgow, Scotland

The actin nucleation promotion factor N-WASP is up-regulated in breast cancer and is coupling pseudopod extension and matrix degradation to facilitate invasive cancer cell migration. Cells migrating through 3D matrices form hybrid adhesion structures termed “actin-hotspots”, which contain N-WASP and display hallmarks of both focal adhesions and invadopodia. We have identified a novel interaction between the N-WASP Interacting Protein, WIP, and the guanine nucleotide exchange factor ARHGEF7/bPIX. ARHGEF7 localises to actin-hotspots in 3D matrices. Loss of ARHGEF7 abolished cancer cell invasion, but increased matrix degradation and pseudopod extension in collagen matrices. These seemingly contradictory results can be explained by our finding that ARHGEF7 is part of a N-WASP/WIP↔ARHGEF7↔PAK2 signalling cascade that is required for adhesion site turnover and force coupling. Loss of ARHGEF7 leads to less tension being applied by actin on adhesion sites as well as the nuclear envelope, resulting in a loss of nuclear movement and subsequently cell motility. The nucleus can act as limiting factor in 3D cell migration. Migrating cells need to actively squeeze the nucleus through matrix pores. Knockdown of nuclear envelope actin binding proteins called Nesprin-1 and -2 severely affect 3D cell migration. We have found a reciprocal regulation of Nesprin-2 and ARHGEF7 function. We here show, using a novel nuclear membrane FRET/FLIM force biosensor, that direct force coupling from actin-hotspots to the nuclear membrane is required for 3D cell migration and we propose that this could be the mechanism to establish polarity during 3D cell migration.

P521**Board Number: B669****Extracellular mechanical cues drive vinculin mediated PI3-kinase signaling to enhance cell invasion in 3D.**M. Rubashkin¹, L. Cassereau¹, R. Bainer¹, C. DuFort¹, Y. Yui¹, G. Ou¹, M.J. Paszek^{2,3}, M.W. Davidson⁴, Y. Chen⁵, V.M. Weaver⁶;¹Bioengineering, University of California, San Francisco, San Francisco, CA, ²Field of Biophysics, Cornell University, Ithaca, NY, ³Kavli Institute at Cornell for Nanoscale Science, Cornell University, Ithaca, NY, ⁴National High Magnetic Field Laboratory and Department of Biological Science, Florida State University, Tallahassee, FL, ⁵Pathology, University of California, San Francisco, San Francisco, CA, ⁶Center for Tissue Engineering and Regenerative Medicine, University of California, San Francisco, San Francisco, CA

Extracellular matrix stiffness induces focal adhesion assembly to drive malignant transformation and tumor metastasis. However, it is unclear how force alters focal adhesions to promote tumor progression. We explored the role of the focal adhesion protein vinculin, a force-activated mechano-transducer, in mammary epithelial tissue transformation and invasion. We found that extracellular matrix stiffness stabilizes the assembly of a vinculin-talin-actin scaffolding complex that facilitates PI3-kinase mediated phosphatidylinositol (3,4,5)-triphosphate phosphorylation. Using defined two and three dimensional matrices, a mouse model of mammary tumorigenesis with vinculin mutants, and a novel super resolution imaging approach, we established that ECM stiffness promotes the malignant progression of a mammary epithelium by activating and stabilizing vinculin and enhancing Akt signaling at focal adhesions. Our studies also revealed that vinculin strongly co-localizes with activated Akt at the invasive border of human breast tumors, where the ECM is stiffest and mechano-signaling is enhanced. Thus, extracellular matrix stiffness could induce tumor progression by promoting the assembly of signaling scaffolds; a conclusion underscored by the significant association we observed between focal adhesion plaque proteins and malignant transformation across multiple types of solid cancer.

P522

Board Number: B670

The local 3D microenvironment controls adhesion dynamics and cell motility by balancing forces at the leading edge.

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Integrin-based adhesions to the surrounding extracellular matrix (ECM) are sites of cellular mechanotransduction, where physical forces are propagated to and from the local microenvironment. It is well established that the physical attributes of simple two-dimensional (2D) ECMs, such as ECM rigidity and ligand density, can modify cell migration through control of cell adhesions; however, it remains unclear how local differences in a complex three-dimensional (3D) ECM can affect cell adhesion mechanics involved in migration. We generated 3D collagen gels with different matrix architectures to identify if ECM-dependent factors, such as matrix topography and rigidity, impact cell migration and adhesion dynamics. Gels ranged from a homogeneous highly reticular (HR) network of thin fibers to a heterogeneous fibrillar-bundled (FB) matrix of loose, parallel-bundled fibers. Atomic Force Microscopy (AFM) revealed that while overall global ECM stiffness was relatively similar between conditions, the parallel-bundled fibers in FB gels demonstrated a local 5-fold greater stiffness. Using live-cell 3D spinning-disk microscopy and automated tracking of EYFP-paxillin containing adhesions, we find that these local differences in matrix stiffness lead to increased 3D adhesion stability, reduced adhesion mobility, and slower adhesion protein turnover in matrices containing bundled fibers, indicating a higher degree of mechanical coupling between the cell and bundled fibers. Lower adhesion stability in HR gels is associated with an increased population of adhesions undergoing retraction at the leading edge, suggesting that cellular contractile forces are not balanced with the local ECM stiffness. By experimentally reducing cellular contractile forces or increasing integrin avidity for the ECM, cell/ECM

interactions become balanced, as shown by increased 3D adhesion stabilization and an increase in cell velocity. Besides establishing that local ECM stiffness is correlated with adhesion stability, we identify a unique process involving contraction of the actin cytoskeleton between adhesions at the leading edge and those proximal to the cell body, resulting in a “pinching” of the matrix. This pinching mechanism is common to all collagen gels and may be important to allow sensing of local matrix stiffness during 3D migration. These results establish that stiff elements of local ECM architecture can stabilize 3D adhesions, increasing the effectiveness of the mechanotransduction machinery required for efficient cell migration in 3D microenvironments.

P523

Board Number: B671

Myosin II pulls the nucleus forward to increase intracellular pressure and drive 3D cell movement.

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Cells use actomyosin contractility to move through three-dimensional (3D) extracellular matrix. Contractility also governs the type of protrusions used during integrin-dependent 3D migration by controlling the switch between lobopodia and lamellipodia. To test the hypothesis that contractility controls intracellular pressure to dictate the mode of 3D cell migration, we directly measured the hydraulic pressure exerted by the cytoplasm in primary human fibroblasts migrating using either lamellipodial or lobopodial protrusions. We found that lobopodia are high-pressure protrusions, and inhibiting contractility switches cells to low-pressure lamellipodia-based movement. In lobopodial cells, the nucleus and associated intracellular membranes physically divide the cytoplasm into high and low pressure forward and rear compartments, respectively. The compartmentalized elevated pressure in lobopodial cells was associated with a polarized distribution of vimentin intermediate filaments and myosin IIA in front of the nucleus and the ability of the nucleus to accelerate periodically away from the trailing edge. Vimentin formed a contractility-dependent complex with actomyosin and the nucleoskeleton-cytoskeleton linker protein nesprin 3. Knocking-down nesprin 3 abolished the independent movement of the nucleus and slowed 3D migration in a manner similar to treatment with blebbistatin. Additionally, nesprin 3 knock-down reduced and equalized intracellular pressure between the front and back cytoplasmic compartments and switched cells to lamellipodia-based migration. Inhibition of myosin II in front of the nucleus by the local application of blebbistatin immediately reduced hydraulic pressure, whereas applying the drug behind the nucleus had no effect. Hence, the nucleus can act as a piston that is pulled forward by actomyosin contractility, acting on nesprin 3 to increase the hydrostatic pressure between the nucleus and the leading edge to drive lamellipodia-independent 3D cell migration. This mechanism is required for the efficient migration of primary human cells through physiological 3D matrix and highlights the importance of physical factors such as matrix microenvironment and pressure in mammalian cellular functions.

P524**Board Number: B672****Cytoskeletal integrity and acto-myosin contractility act by distinct pathways to regulate nuclear Yap1 localization and phosphorylation during mechanotransduction.**A. Das¹, C. Waterman²;¹Cell biology and physiology, National Institutes of Health/NHLBI, Bethesda, MD, ²National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD

Mechano-transduction is key to important biological processes such as gastrulation, vascularization and even cancer metastasis. Recent discoveries connect cellular mechanosensing to global transcriptional regulation, where the transcriptional co-activator Yap1/Taz induces transcription of target genes upon increased cellular tension induced by actomyosin contractility on stiff ECMs. Yap1/Taz regulation is also well-characterized in the context of contact inhibition-mediated Hippo signaling, where the dephosphorylation of S127 promotes its nuclear import and activity. However, the relationship between phosphorylation and nuclear import during mechano-regulation of Yap1/Taz is less clear. Here we sought to dissect the relationships between cytoskeletal integrity, contractility, Yap1 localization and Yap1 phosphorylation. We first examined the relationships between cytoskeletal integrity and either Yap1 localization or phosphorylation. We utilized the F:G actin ratio as a proxy for cytoskeletal integrity. Biochemical fractionation and single cell imaging assays showed that treatment with Latrunculin A (LatA) or plating cells on soft substrates decreased the F:G actin ratio, while either treatment with the actin stabilizer jasplakinolide (jasp), inhibition of myosin II with blebbistatin, or plating cells on stiff substrates increased the F:G actin ratio. We then examined the correlations between F:G actin ratio and either Yap1 localization or phosphorylation across these conditions. This showed that F:G actin ratio correlated positively with nuclear localization, but not with phosphorylation of Yap1. Specifically, compared to control, LatA and soft substrates reduced Yap1 nuclear localization and increased its phosphorylation, while Jasp, blebbistatin, and stiff substrates increased Yap1 nuclear localization compared to LatA, but had mixed effects on its phosphorylation. We then examined the relationship between contractility and Yap1 phosphorylation by utilizing the collagen gel contraction assay. This showed that LatA, Jasp, and blebbistatin, all of which induce Yap1 phosphorylation, also reduce contractility, indicating that contractility negatively correlates with Yap1 phosphorylation. Biochemical fractionation of nucleus and cytoplasm further revealed that conditions with high F/G actin ratio could even enrich phosphorylated Yap1 in the nucleus, indicating that cytoskeletal integrity is dominant in regulation of Yap1 nuclear localization, independent of its phosphorylation. Hence this study indicates a novel mechanism where cytoskeletal integrity and acto-myosin contractility act by distinct pathways to regulate nuclear Yap1 localization and phosphorylation during mechanotransduction.

P525**Board Number: B673****Actin dynamics and calcium signaling in B cells respond to surface topography.**C.M. Ketchum¹, X. Sun², H. Miller³, J. Fourkas⁴, W. Song³, A. Upadhyaya⁵;¹Biophysics Program, University of Maryland, College Park, MD, ²Department of Chemistry and Biochemistry, University of Maryland, College Park, MD, ³Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD, ⁴Dept of Chemistry and Biochemistry, University of Maryland, College Park, MD, ⁵Physics and IPST, University of Maryland, College Park, MD

B cells become activated by membrane-bound antigen binding to the B cell receptor (BCR), inducing actin dynamics, reorganization of receptors into microclusters, and cell spreading. In vivo B cells gather antigen from a variety of sources which may have different physical characteristics such as mobility, stiffness or topography. However, the effect of these parameters on BCR clustering and signaling activation is not understood. Here we have studied the role of topography in cell spreading, actin polymerization and signaling activation. BCR ligand coated substrates presenting ridges of variable spacing were used to probe the interaction of B cells with non-planar surfaces. Using high-resolution TIRF and confocal microscopy of live cells, we followed the movement of BCR clusters and the dynamics of actin. Ridge separations of less than 1.5 μ m induced actin waves that travel parallel to the ridges, resulting in protrusions and retractions of the cell edge. Separations of 3 μ m or greater result in large-scale contractions of the actin network. B cells are known to exhibit periodic calcium enrichment after antigen engagement. On flat substrates we have measure the period to be \sim 30s, consistent with previously observed values. Interestingly, we found the period of the calcium enrichment was dependent on ridge spacing, with increasing time intervals on smaller spacings. Periodic calcium enrichment was also found to be slower on flat substrates when affected by actin-inhibiting drugs, such as latrunculin. Our results indicate that B cells are sensitive to topographical features, resulting in modulated actin dynamics and that calcium signaling is coupled to substrate-proximal actin dynamics.

P526**Board Number: B674****Traction Forces of Primary Human Macrophages.**L.E. Hind¹, M. Dembo², D.A. Hammer^{3,4};¹University of Pennsylvania, Philadelphia, PA, ²Boston University, Boston, MA, ³Department of Chemical and Biomolecular Engineering, University of Pennsylvania, Philadelphia, PA, ⁴Department of Bioengineering, University of Pennsylvania, Philadelphia, PA

Macrophages play a crucial role in our bodies as first responders in the innate immune system. In order to properly function they must migrate efficiently to sites of infection and inflammation. The signaling mechanisms underlying macrophage migration have been previously studied but to date no one has investigated the mechanical mechanism of macrophage migration. Using traction force microscopy, we have created the first traction maps of primary human macrophages migrating on compliant

polyacrylamide gels. We found that the force generated by macrophages is dependent on the stiffness of their underlying substrate with cells on stiff gels producing greater forces than cells on soft gels. Traction maps of motile macrophages have shown that macrophages produce their highest forces in the leading edge of the cell relative to cell motion indicating that they use a forward-towing mode of migration. This type of motility is common among mesenchymal cells and dendritic cells, another monocytic leukocyte, but the reverse of what was seen for neutrophils which propel themselves forward through a rearward squeezing mechanism. These varied results illustrate the importance of investigating the force profiles and signaling pathways for each individual cell type. We used a variety of chemical inhibitors to determine which signaling molecules are crucial for force generation in macrophages. The inhibitors Blebbistatin, Y27632, and LY294002 which block myosin II, the RhoA kinase ROCK, and PI3K signaling respectively all caused a significant reduction in macrophage force generation. This indicates that, like in most cells, myosin contraction through RhoA is necessary for force generation. PI3K is known to activate a variety of pathways at the leading edge of migrating cells so we decided to further investigate the necessity of one of its downstream effectors, Rac. We found that inhibition of Rac by NSC23766 which blocks signaling of the Rac GEF Tiam1 did not significantly change macrophage force generation. However, inhibition of Rac by 6-thio-GTP which blocks Rac binding to its GEF Vav1 leads to a significant reduction in force. This indicates that Rac is necessary for force generation downstream of some but not all Rac GEFs, illustrating the complexity of the signaling networks involved in force generation.

P527

Board Number: B675

Investigating the role of type IV pili mechanics and force production in pathogenic *Neisseria gonorrhoeae* and commensal *Neisseria elongata* during their interaction with human host cells.

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Type IV pili (Tfp) are long polymeric bacterial fibers produced on the surface of a wide variety of bacteria and archaea. In *N. gonorrhoeae* (Ng) and *N. elongata* (Ne) Tfp are involved in adhesion and motility, DNA uptake, and have essential roles in biofilm formation and virulence in the case of Ng. Tfp are dynamic and can generate pulling forces as high as 1 nN. We investigate the role of Tfp retraction and force production in the infection process using genetic methods, fluorescence microscopy and magnetic tweezers. We generated various mutant bacterial strains in both Ng and Ne with deleted components of the Tfp retraction machinery, i.e. pilT, the main protein responsible for Tfp retraction, and two other associated motors, pilT2 and pilU. We also generated Ng and Ne strains where the pilT gene, the pilT promoter, or both were switched with that of Ne and Ng, respectively. A431 human epithelial cells were directly infected with the mutant bacteria and actin recruitment at the infection site was monitored. We also simulated the infection by allowing Tfp-coated magnetic beads to interact with the human cells and subjecting the beads to variable magnetic forces to mimic Tfp pulling. Direct measurement of force

production by Tfp retraction in bacteria was also recorded using flexible polyacrylamide micro-pillar patterns. Preliminary results show that i) the Tfp retraction mechanics differs between Ng and Ne strains and it depends on pilT and pilT2 motors, ii) PilT2 deletion decreases Tfp retraction speed by half in *N. gonorrhoeae*, as previously reported, and has a similar effect in *N. elongata*, iii) actin recruitment in human cells at the infection site correlates with the pulling force. Our investigation promises to elucidate the role of the Tfp retraction machinery and the forces exerted by Tfp during bacterial interaction with human cells.

P528

Board Number: B676

The dynamic interplay between cleavage furrow proteins as a model for cellular mechanoresponsiveness.

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Cell shape changes associated with processes such as cytokinesis and motility take place on tens of seconds time-scale, but are derived from molecular events occurring much more quickly, such as dynamic protein-protein interactions, filament assembly and rearrangement, and force generation by molecular motors. While the local accumulation of cytoskeletal elements can often be driven by signaling pathways, mechanical stresses also direct protein accumulation. A myosin II-based mechanosensory system, which controls cellular contractility and cell shape change during cytokinesis and under applied stress, was previously identified in *Dictyostelium*. This system tunes myosin II accumulation by feedback through the actin network, particularly the actin crosslinker cortexillin I. The cortexillin-binding IQGAP proteins served as major regulators of the system. Here, we examine the dynamic interplay between these key cytoskeletal proteins using fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS), defining the short time-scale turnover characteristics of these players during cytokinesis or in response to mechanical stresses. We found that the actin network is highly dynamic, and turns over within 0.7 seconds post-bleach. Polar cortex enriched crosslinkers also show sub-second turnover at the cleavage furrow, while equatorially enriched proteins such as cortexillin I, IQGAP2 and myosin II turn over much more slowly on the 1-5 s time-scale. As compared to the interphase cortex, the mobility of the equatorial proteins is greatly reduced at the cleavage furrow, likely driving their accumulation at this region. This mobility shift does not arise from a single biochemical event, but rather from a global inhibition in protein dynamics by mechanical stress-associated changes in the cytoskeletal structure. We also expanded our genetic findings using a proteomics approach, which demonstrated that myosin II interacts with both cortexillin I and IQGAP2. This experiment was performed in the presence of Mg²⁺ and ATP to ensure that these proteins were not simply interacting through a common actin filament. We are now using two-color FCS

in tandem with in vivo biochemistry to further characterize the myosin II-cortexillin I-IQGAP2 interaction and determine how their molecular interplay leads to mechanosensation. Our results indicate that high crosstalk occurs between the different components of the mechanosensory system under stress. This mechanical tuning of contractile protein dynamics provides robustness to the cytoskeletal framework responsible for regulating cell shape, and contributes to the fidelity of cytokinesis.

P529

Board Number: B677

In vivo monitoring of calcium signaling at the single-cell and tissue levels in the spermatheca of *C. elegans*.

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The spermatheca is a stretchy and contractile tube in the reproductive system of the nematode *C. elegans* which is composed of a 24-cell tube and a 4-cell syncytial valve. The spermatheca is stretched by oocyte entry, remains stretched for a regulated period of time, then forcefully contracts to expel the fertilized egg. Previous work in the lab has examined calcium signaling at the tissue level during ovulation events, using widefield microscopy to image fluorescence from the genetically encoded calcium sensor GCaMP [1]. To further dissect the tissue level calcium signaling, and to develop an understanding of how calcium signaling in individual cells generates a tissue level response, we developed transgenic constructs to visualize the individual cells in the tissue. We utilized CRISPR genome editing [2] to generate stable lines of transgenic worms expressing an INX-12::mApple fusion protein that labels the junctions between the spermathecal cells in red. Combining these two fluorescent biosensors, we generated a worm strain where the spermathecae are labeled for calcium in green and cell boundaries in red.

Using a laser scanning confocal microscope to image ovulation events in live, intact animals, we optimized imaging parameters to enable fast, simultaneous acquisition of calcium signaling in the green channel and cell boundaries in the red channel. These fast acquisitions enable us to capture ZT stacks of the tissue, allowing the observation of cells in four dimensions during ovulation events. Preliminary evidence suggests that calcium signaling can differ markedly between adjacent spermathecal cells during ovulation events, with some individual spermathecal cells exhibiting very high levels of calcium signaling while adjacent cells exhibit considerably lower levels. These preliminary results indicate the utility of this strain for detailed investigation of calcium signaling in the spermatheca at the single-cell and tissue levels. This work will advance our understanding of how tissue level responses are generated in tubular arrangements of cells responding to stretch.

[1] I. Kovacevic, J. M. Orozco, and E. J. Cram, "Filamin and phospholipase C- ϵ are required for calcium signaling in the *Caenorhabditis elegans* spermatheca.," *PLoS Genet.*, vol. 9, no. 5, p. e1003510, May 2013.

[2] A. E. Friedland, Y. B. Tzur, K. M. Esvelt, M. P. Colaiácovo, G. M. Church, and J. a Calarco, "Heritable genome editing in *C. elegans* via a CRISPR-Cas9 system.," *Nat. Methods*, vol. 10, no. 8, pp. 741–3, Aug. 2013.

P530

Board Number: B678

Cell migration in mechanically resistive environment.

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The mechanical environment of a cell influences its migration. It has been observed in particular that certain cell types such as *Dictyostelium discoideum* and cancer cells tend to migrate by forming blebs instead of pseudopodia/lamellipodia in mechanically resistive environments^{1–3}. However, very little is known about the mechanisms governing this transition between different modes of migration^{4,5}. In this work, we aim to disentangle the contributions of two key mechanical factors that might trigger this switch, the environment's stiffness (the extent of local deformation of extra-cellular matrix by the cell) and its state of stress (amount of pre-existing tension or compression). We use *Dictyostelium discoideum* as a model to study these questions. A custom built device, the "cell squasher", is used to compress an agarose gel of known stiffness against a glass cover slip under a dynamically controlled and uniform loading condition. A chemotactic gradient (using cAMP) is used to direct the cells under the agarose while monitoring cell's migration and recording its morphology and trajectory. Our preliminary results indicate that switching to bleb mode of migration can be triggered by an increase of either the external loading or the gel stiffness. This transition is also associated with a decrease of cell speed. Increasing the load even further triggers the rounding up of the cells and dramatically slows down the retraction of blebs; migration ceased in most of the cases. The setup is currently applied to study the dynamics of the lamellipodia/bleb transition. This work may be of significant importance in understanding the initiation of cancer metastasis where cells often migrate using blebs in environments often highly deformed by the growth of the tumour⁵.

References:

1. Zatulovskiy, E., Tyson, R., Bretschneider, T. & Kay, R. R. Bleb-driven chemotaxis of *Dictyostelium* cells. *J. Cell Biol.* 204, 1027–44 (2014).
2. Charras, G. & Paluch, E. Blebs lead the way: how to migrate without lamellipodia. *Nat. Rev. Mol. Cell Biol.* 9, 730–6 (2008).

3. Tyson, R. A., Zatulovskiy, E., Kay, R. R. & Bretschneider, T. How blebs and pseudopods cooperate during chemotaxis. *Proc. Natl. Acad. Sci.* 1322291111– (2014). doi:10.1073/pnas.1322291111
4. Bergert, M., Chandradoss, S. D., Desai, R. A. & Paluch, E. Cell mechanics control rapid transitions between blebs and lamellipodia during migration. *Proc. Natl. Acad. Sci. U. S. A.* 109, 14434–9 (2012).
5. Tozluoğlu, M. et al. Matrix geometry determines optimal cancer cell migration strategy and modulates response to interventions. *Nat. Cell Biol.* 15, 1–14 (2013).

P531

Board Number: B679

Fibroblasts use Filopodia Extensions to Probe Substrate Rigidity before Occupying an Area.

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Rigidity sensing and durotaxis are thought to be important elements in wound healing, tissue formation, and cancer treatment. It has been challenging, however, to study the underlying mechanism due to difficulties in capturing cells during the transient response to a rigidity interface. We have addressed this problem by developing a model experimental system that confines cells to a micropatterned area with a rigidity border. The system consists of a rigid domain of one large adhesive island, adjacent to a soft domain of small adhesive islands grafted on non-adhesive soft gels. This configuration allowed us to test rigidity sensing away from the cell body during probing and spreading. NIH 3T3 cells responded to the micropatterned rigidity border similarly to cells at a conventional rigidity border, by showing a strong preference for staying on the rigid side. Furthermore, cells used filopodia extensions to probe substrate rigidity at a distance in front of the leading edge and regulated their responses based on the strain of the intervening substrate. Soft substrates inhibited focal adhesion maturation and promoted cell retraction, while rigid substrates supported stable adhesions and cell spreading. Myosin II was required for not only the generation of probing forces but also the retraction of probing structures in response to soft substrates. We suggest that a myosin II-driven, filopodia-based probing mechanism ahead of the leading edge allows cells to migrate efficiently, by sensing physical characteristics before moving over a substrate to avoid back-tracking.

P532**Board Number: B680****Dynamic tensile forces drive collective migration through three-dimensional extracellular matrices.**A. Piotrowski¹, N. Gjorevski¹, V. Varner¹, C. Nelson^{1,2};¹Chemical and Biological Engineering, Princeton University, Princeton, NJ, ²Dept. of Molecular Biology, Princeton University, Princeton, NJ

Collective migration drives a variety of biological processes, including wound healing, angiogenesis, branching morphogenesis, and cancer invasion. While collective migration is thought to be coordinated by soluble factors, it is also a fundamentally physical process. We used arrays of microfabricated tissues to elucidate the physical mechanisms by which epithelial cells move collectively through three-dimensional (3D) extracellular matrix (ECM). Cells undergo collective invasion predictably from specific locations within the microfabricated tissues, allowing for high-throughput analysis and manipulation of mechanical parameters. We examined the interactions between migrating cells and their surrounding ECM, as well as the dynamics of migrating cohorts. Using traction force microscopy, we found that cells exert tensile forces on their surrounding ECM while undergoing collective migration. In addition to serving a physical role in propelling cells forward, these forces also stimulated mechanotransduction signaling in the cells at the leading edge of the migrating cohort. Confocal reflectance microscopy revealed that the matrix ahead of the migrating cohort was remodeled into highly aligned fiber bundles along which the cohort preferentially migrated. Moreover, timelapse imaging showed that cohort extension was highly dynamic, and that variations in cohort length were highly correlated and in phase with deformations and tensile forces in the ECM. These results suggest both mechanical and signaling roles for dynamic tensile forces during collective migration.

P533**Board Number: B681****The dendritic cell cytoskeleton regulates ICAM-1 clustering and lateral mobility to promote T cell adhesion and activation.**W. Comrie¹, S. Li², S. Boyle², J.K. Burkhardt³;¹University of Pennsylvania, Philadelphia, PA, ²Children's Hospital of Philadelphia, Philadelphia, PA,³Department of Pathology and Laboratory Medicine, The Children's Hospital of Philadelphia Research Institute, Philadelphia, PA

Integrity of the dendritic cell (DC) actin cytoskeleton is essential for T cell priming, but the underlying mechanisms are poorly understood. We show that the DC F-actin network regulates the lateral mobility of ICAM-1, but not MHCII. ICAM-1 mobility and clustering is regulated by maturation-induced changes in the expression and activation of moesin and α -actinin-1, which associate with actin filaments and the ICAM-1 cytoplasmic domain. Constrained ICAM-1 mobility is important for DC function, since DCs

expressing a high mobility ICAM-1 mutant lacking the cytoplasmic domain exhibit diminished antigen-dependent conjugate formation and T cell priming. These defects are associated with inefficient induction of LFA-1 affinity maturation, consistent with a model in which constrained ICAM-1 mobility opposes forces on LFA-1 exerted by the T cell cytoskeleton. Our results reveal a novel mechanism through which the DC cytoskeleton regulates mechanotransduction at the immunological synapse.

P534

Board Number: B682

F-actin flow drives affinity maturation and spatial organization of LFA-1 at the immunological synapse.

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Integrin-dependent interactions between T cells and antigen presenting cells are vital for proper T cell activation, effector function, and memory. Regulation of integrin function occurs via conformational change, which modulates ligand affinity, and receptor clustering, which modulates valency. Here, we show that conformational intermediates of LFA-1 form a concentric array at the immunological synapse. Using an inhibitor cocktail to arrest F-actin dynamics, we show that organization of this array depends on F-actin flow and ligand mobility. Furthermore, F-actin flow is critical for maintaining the high affinity conformation of LFA-1 and for enhancing valency through recruitment of LFA-1 to the cell-cell interface. Finally, we show that F-actin forces are opposed by immobilized ICAM-1, which triggers LFA-1 activation through a combination of induced fit and tension-based mechanisms. Our data provide direct support for a model in which the T cell actin network generates mechanical forces that regulate LFA-1 activity at the immunological synapse.

P535

Board Number: B683

The Role of Annexin A4 in Stabilizing Cell Membranes Undergoing Biomechanical Stress.

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Biomechanical stress due to increased intraocular pressure (IOP) is associated with inner retinal pathology characterized by glial reactivity and retinal ganglion cell death. However, the

mechanotransduction mechanism by which resident cells respond to this stress is not understood. We have developed an *in vitro* model whereby controlled magnitudes of pathologically relevant stress are generated in retinal astrocytes to mimic the conditions that occur *in vivo* during the elevation of IOP. Proteomic analysis has identified Annexin A4, (ANXA4), a Ca^{2+} -dependent phospholipid binding protein, to be significantly elevated in this model. Relatively little is known about the function of ANXA4, but it plays a role in membrane interactions by contributing to their rigidity through calcium dependent translocation. We report that ANXA4 responds to Ca^{2+} and biomechanical stress in a way that can increase cell survival or inhibit pro-apoptotic pathways. Immunofluorescence microscopy was used to identify the pattern of ANXA4 protein in normal mouse, rat, and human retinas. Acute elevation of IOP was induced at the retina *in vivo* followed by quantitative RT-PCR. Live cell imaging of an astrocytic cell line was used to visualize ANXA4 localization and membrane dynamics in response to increased biomechanical stress and elevated intracellular Ca^{2+} . ANXA4 immunofluorescence staining revealed conserved localization to the ganglion cell layer and optic nerve head in normal mouse, rat, and human retinas. Furthermore, there was a 6-fold increase in ANXA4 mRNA expression by 18 hours following elevation of IOP *in vivo* ($p=0.039$). Live cell imaging showed evidence of ANXA4 localization to cellular and nuclear membranes upon increases in intracellular Ca^{2+} and dramatically reduced membrane blebbing. These results show that ANXA4 protein is localized to the cells lining the inner retina and optic nerve head and that it responds to biomechanical and Ca^{2+} stress. They further suggest that ANXA4 can increase cell integrity by inhibiting membrane blebbing when translocated to improve cell survival under conditions of biomechanical stress.

P536

Board Number: B684

The CD2 isoform of protocadherin-15 is an essential component of the tip-links in mature auditory hair cells.

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Protocadherin-15 (Pcdh15) is a component of the tip-links, the extracellular filaments that gate mechano-electrical transduction channels of the inner ear hair cells. There are three Pcdh15 splice isoforms (Pcdh15-CD1, Pcdh15-CD2, Pcdh15-CD3), which only differ by their cytoplasmic domains. These three isoforms are thought to function redundantly in mechano-electrical transduction during hair-bundle development, but whether any of these isoforms composes the tip-link in mature hair cells was still unknown. By immunolabeling we showed that Pcdh15-CD2 is correctly localized in mature auditory

hair cells to form the lower tip-link component. We generated postnatal hair cell-specific conditional knockout mice that lose only Pcdh15-CD2 after normal hair-bundle development. Electrophysiological analysis of these mice indicated that mechano-electrical transduction is lost from P21 onwards. Morphological analysis of the auditory hair cells of these mice proved that the loss of mechano-electrical transduction is due to the loss of tip-links. We conclude that this isoform is an essential component of the tip-links in mature murine auditory hair cells. This conclusion could be extended to humans, since a frame-shift mutation in PCDH15 (in the homozygous or compound heterozygous state) that only affects Pcdh15-CD2 was identified in profoundly deaf children from two unrelated families. These results provide key information for the identification of new components of the mature auditory mechano-electrical transduction machinery.

P537

Board Number: B685

Morphodynamic analysis of T lymphocyte migration in confined microenvironments.

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Leukocytes such as dendritic cells (DCs) or T lymphocytes are highly plastic and undergo a variety of molecular interactions with the substrate and morphological changes to efficiently move inside the organism. Although most leukocytes are able to migrate in the absence of integrins, they use, by default, the classical translocation mode: at the leading edge actin polymerization pushes forward, integrin-dependent adhesions couple the substrate to the retrograde actin flow, and actomyosin contraction allows the uropod to retract and push the nucleus forward. To decipher the role of adhesion in leukocyte locomotion, we set-up a PDMS device (Le Berre et al., 2014) that allows for real time microscopy combined with variable degrees of confinement between 2 parallel surfaces. In addition substrate micropatterning permits creation of defined adhesive domains. TIRF microscopy revealed that lamellipodia of confined DCs and T cells only occasionally and punctually contact the substrate and that these contacts are not associated with forward translocation. This indicates that actual force transduction occurs at the cellular mid-zone, while lamellipodial adhesions are dispensable for locomotion. Under non-adhesive (PLL-PEG-coated) conditions both T cells and DCs showed extensive retrograde actin flow. However, only DCs were able to migrate (Renkawitz et al., 2009), while T cells “ran on the spot”, indicating fundamental differences in the mechanisms how the two cell types generate friction with the substrate. In addition patterned structures alternating adhesive and non-adhesive areas induce almost instantaneous switch between polarized motility and loss of polarity associated with blebbing. Finally we are quantifying morphometric parameters from both the actin cytoskeleton and the nucleus together with velocity to decipher how migrating leukocytes can dynamically reorganize their actomyosin machinery in response to adhesive cues.

Intermediate Filaments

P538

Board Number: B686

Repair cells activated in response to wound healing exhibit invasive function.

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Tumors are often referred to as “wounds that do not heal” because of their resemblance to a tumor cell environment that never resolves. This phenomenon suggests that tumor formation may reflect an aberrant wound healing process. In fact, fibrosis, a negative outcome of wound repair, is believed to increase susceptibility to tumor formation. Using a mock cataract surgery wound healing model in which we are able to follow the response of an epithelium to a physiologically relevant wounding, we have identified a subpopulation of vimentin-rich mesenchymal cells with repair function that are endogenous residents of an unwounded epithelium. Repair cells are activated to migrate to the wound edge immediately upon injury, serving as the directors of the wound healing process by guiding the collective movement of a wounded epithelium across the denuded (injured) basement membrane. It is not fully understood how the repair cells function to regulate healing of the epithelium; however, we have shown that vimentin function is central to their migration. Further elucidation of the migratory properties of repair cells and how these cells perform their role as the immediate responders to injury to coordinate the wound healing is important not only to understand their function in wound healing but also how they might contribute to disease states such as cancer. We now report that vimentin-rich repair cells at the wound edge are enriched for CD44, MMP-2, and MMP-9, molecules known to mediate interaction with, and/or remodeling of, the ECM microenvironment. These properties could provide repair cells with the ability to navigate their microenvironment. Furthermore, we discovered that vimentin-rich repair cells have innate invasive properties. These cells are able to invade through an extracellular matrix environment in both matrigel transwell and 3D matrigel invasion assays. Repair cell invasion appeared to occur in a vimentin-dependent manner since treatment with the vimentin inhibitor Withaferin A suppressed repair cell invasion. Understanding the wound healing process that results from a clinically relevant wounding can elucidate mechanisms that activate an invasive program where repair cells direct collective migration of epithelial cells, and is a pivotal step forward toward understanding how this repair cell function can become dysregulated and hijacked to promote disease.

P539**Board Number: B687****The Role of O-linked N-acetylglucosamine on Vimentin Function.**H.J. Tarbet¹, T. Smith², M. Boyce³;¹Department of Biochemistry, Duke University, Durham, NC, ²Duke University, Durham, NC, ³Dept. of Biochemistry, Duke University Medical Center, Durham, NC

Protein modification by O-linked β -N-acetylglucosamine (O-GlcNAc) is an essential intracellular signaling mechanism that affects diverse processes such as cell metabolism, cycle, and death. Aberrant signaling has been implicated in numerous human diseases, including cancer, diabetes, and neurodegeneration. However, major aspects of O-GlcNAc signaling are poorly understood, including the functional consequences of these modifications on proteins. Based on recent literature and our own preliminary results, our lab proposes that one important function of O-GlcNAc is mediating protein-protein interactions. In collaboration with the Kohler group at UT Southwestern, we previously developed a method of covalently capturing proteins that interact through O-GlcNAc using an analog with a diazirine photocrosslinking moiety. We used this approach to identify several proteins that engage in O-GlcNAc-mediated protein-protein interactions, including vimentin, an intermediate filament (IF) protein known to be O-GlcNAcylated. Vimentin is important in the integrity of mesenchymal cells and has been implicated in tissue movement and various cancers. Although vimentin glycosylation was identified in 2008, the purpose remains unknown. Due to vimentin's involvement in cell motility and the Epithelial to Mesenchymal Transition, we hypothesized that O-GlcNAc may be important in regulating vimentin function in these processes. Here we show that mutations in known O-GlcNAcylation sites drastically reduce O-GlcNAc-mediated protein-protein interactions of vimentin identified through our assay. In addition, we have characterized these mutants in an array of phenotypic assays such as wound healing, migration, and cell cycle transitions, and have examined how IF formation is regulated by O-GlcNAcylation. Many IF proteins besides vimentin are also glycosylated, so our work may provide broad insight into how O-GlcNAc regulates IFs in general.

P540**Board Number: B688****Vimentin is a key regulator of repair cell function in wound healing and fibrotic disease.**J.L. Walker¹, B.M. Bleaken¹, L. Zhang², S. Menko¹;¹Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, PA, ²Anatomy, Thomas Jefferson University, Philadelphia, PA

Wound healing of an epithelium involves the collective movement of the injured epithelium, directed at the wound edge by a population of mesenchymal leader cells. To study wound repair and regeneration of epithelial tissues we developed an ex vivo, clinically relevant wound model, which involves mock cataract surgery of isolated embryonic chick lenses. With this model we discovered that the

mesenchymal leader cells were, in fact, the progeny of an innate subpopulation of vimentin-rich repair cells. Vimentin was essential to the movement of the repair cells along the exposed basement membrane at the wound edge, as well as to the function of the repair cells in directing wound-healing of the epithelium. In the uninjured lens the vimentin-rich repair cell progenitors are located in niches among the epithelium. These cells respond rapidly to injury, quickly expanding in cell number and migrating to the leading edge of the wound where they extend vimentin-rich, actin-poor lamellipodial extensions, where it is the vimentin filaments that link to paxillin-containing integrin focal adhesion contacts in the lamellipodial tips. These findings highlight a key role for vimentin intermediate filaments rather than actin filaments in regulating migration and repair cell function during wound healing. Changes in vimentin detergent solubility, reflective of different vimentin assembly states, were found associated with migration. Non filamentous forms of vimentin, associated with detergent soluble fractions, were present near the tips of lamellipodia and an increase in the detergent soluble vimentin pool occurred in the migratory region. These results highlight a potential function for soluble vimentin in regulating migration. In addition to acting as directors of the wound healing process, we found that these vimentin-rich repair cells have a high potential to differentiate into myofibroblasts, the cell type associated with development of fibrotic disease, when they encounter a rigid microenvironment. Treatment with Withaferin A, for which vimentin is a druggable target, suppressed myofibroblast differentiation. Here too, vimentin function in the repair cells appears to be required for their acquisition of a myofibroblast phenotype. Vimentin has emerged as a key regulator of repair cell migration, their function in wound healing, as well as their potential to transition to disease-causing myofibroblast.

P541

Board Number: B689

Microtubule-dependent transport and dynamics of vimentin intermediate filaments.

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We studied two aspects of vimentin intermediate filaments dynamics, transport of filaments and subunit exchange. Using Structured Illumination Microscopy (SIM) of live cells, we directly observed transport of long intermediate filaments at the cell periphery. To study filament transport elsewhere in the cell, we used the photoconvertible protein mEos3.2 fused to vimentin. This technique enabled us to selectively label a subset of filaments within the dense vimentin network by photoconversion of green fluorescence to red in a small area of the cytoplasm. We then followed red filaments by time-lapse Total Internal Reflection Fluorescence Microscopy (TIRFM), which demonstrated that photoconverted filaments are rapidly transported along linear tracks. Filament transport was microtubule-dependent,

likely due to transport by microtubule-based motors. Transport was independent of microtubule dynamics and/or interaction of vimentin with the microtubule plus-tip binding protein APC. We also used mEos3.2-vimentin and photoconversion to study subunit exchange in vimentin filaments over the course of several hours. We found that even 17 hours after photoconversion, photoconverted (red) vimentin formed distinct patches and was not completely intermixed with green vimentin filaments. This pattern was also observed in cells that divided after conversion. These data show that vimentin filaments are stable and do not disassemble into individual subunits even during cell division. Instead, dynamics of intermediate filaments include severing and re-annealing. Together, these results contribute to the understanding of intermediate filament organization, which is important for maintaining mechanical integrity during cell migration. This work was supported by the Intermediate Filament Program Project Grant, NIGMS # P01GM09697.

P542

Board Number: B690

Vimentin filament precursors exchange subunit in an ATP-dependent manner.

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Unit-length filaments (ULFs) are non-polar oligomers formed by the lateral association of eight tetramers of vimentin polypeptides. These oligomers are the basic building blocks that form intermediate filaments by longitudinal annealing. Previous studies have shown that mature intermediate filaments can exchange segments along their length probably through severing and annealing. The long time scale for this process is indicative of the stability of the vimentin polymer as compared to the highly dynamic turnover of microtubules and actin filaments. Here we studied dynamic properties of ULFs using the vimentin mutant Y117L, which does laterally associate into ULFs but fails to longitudinally anneal and thus does not form vimentin filaments. By monitoring the fluorescence of Y117L mutant tagged with the photoconvertible protein EOS3.2, we found that ULFs are highly dynamic. Subunit exchange between ULFs occurred within seconds and is limited by diffusion of vimentin polypeptides in the cytoplasm rather than their association and dissociation from ULFs. Biochemical analysis and microscopy data demonstrate that cells expressing vimentin Y117L mutant contain a large pool of soluble vimentin that is in the rapid equilibrium with ULFs. Furthermore, our study demonstrated that subunit exchange between ULFs requires ATP; ATP depletion causes a dramatic reduction of the soluble vimentin pool. Therefore, this study shows that vimentin filament precursors can be present in two distinct states, the ULF vimentin oligomer and a soluble pool. The fast transition between these two states is an ATP-dependent active process that might play a critical role in the regulation of the first steps of the filament assembly in the cell.

P543**Board Number: B691****Lamellar tissue-specific expression of novel keratins and the effect of laminitis on keratin isoform expression.**

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Equine laminitis is a common and devastating disease that affects the hoof epidermis and its adhesion to underlying dermal connective tissues. Our previous quantitative proteomic analysis identified two novel equine keratins (K), K42 and K124 as the most abundant keratin pair in hoof lamellar tissue. We hypothesize that these unique keratins are lamellar tissue-specific and that laminitis alters keratin isoform expression patterns, thus altering the differentiation phenotype and mechanical properties of epidermal lamellae. The first objective of this study was to use qualitative PCR to determine the tissue expression of keratin isoform genes known to be expressed at the protein level in the equine hoof, skin, and cornea. Epidermal tissue-specific gene expression of the following keratin isoforms was determined by PCR: K42 and K124 are expressed only in hoof lamellar tissue, K3 is expressed only in the ocular cornea, and K10 is expressed only in skin. However, K10 has undergone a gene duplication in the equine genome and a second "K10b" gene copy was detected in all epidermal tissues. Our second objective was to relate keratin isoform protein expression and localization to laminitis histopathology.

Paraformaldehyde-fixed/sucrose dehydrated lamellar tissue cryosections from non-laminitic and laminitic horses were subjected to indirect immunofluorescence of the epidermal basal cell keratin, K14, confocal microscopy, and visual scoring by two independent evaluators. Semi-quantitative protein expression in laminitic and non-laminitic lamellar tissues was evaluated by K14 immunoblotting and image densitometry. K14 was predominantly localized to basal cells only of the secondary epidermal lamellae of non-laminitic tissues and to both basal and suprabasal cells of the secondary epidermal lamellae of tissues with histological evidence of laminitis. Moreover, K14 protein expression was increased in laminitic lamellar tissue. These results suggest that laminitis alters the keratin expression, and hence, differentiation phenotype of secondary epidermal lamellar cells. Ongoing studies include the investigation of K42 and K124 protein expression, localization, and utility as a tissue-specific laminitis biomarker.

This study was supported by the Grayson-Jockey Club Research Foundation, Inc., the Bernice Barbour Foundation, Inc., the University of Pennsylvania Fund for Laminitis Research, the Raymond Firestone Trust, and the National Institutes of Health (T35 RR07065).

P544**Board Number: B692****Phenotypic Assay Development Targeting Gigaxonin-mediated Intermediate Filament Aggregation associated with Giant Axonal Neuropathy (GAN).**M.G. Mendez¹, J. Inglese¹;¹Assay Development and Screening Technology (ADST) Laboratory, NIH/NCATS, Rockville, MD

GAN is a rare autosomal recessive disorder caused by mutations in gigaxonin (also *GAN*), a proposed E3 ligase substrate adaptor. GAN progresses rapidly from initial peripheral nervous system involvement that affects coordination and strength, to systemic central and peripheral motor and sensory deficits.

A hallmark of GAN is abnormally large caliber axons, which represent an increasingly greater proportion of axons as the disease progresses; their myelin sheaths are also greatly reduced. Giant axons contain abnormally densely packed, large masses of neurofilaments that can grow to fill the intracellular space. In astrocytes, fibroblasts and muscle fibers, cell-specific intermediate filaments – including vimentin in fibroblasts – form aggregates as well.

In order to screen large compound libraries for entities capable of reducing the effects of gigaxonin loss, a model gigaxonin-null cell line must be proliferative over many passages and exhibit a phenotype reliably distinguishable within the time, imaging and cell culture constraints of microtiter plate format. For many assays, high efficiency reverse transfectability is also desirable.

We used CRISPR/Cas9 genome editing techniques to generate frameshift mutations and isolated homozygous and heterozygous clones in U-2 OS cells. Mutations were confirmed by sequencing.

In contrast to the normal vimentin network distribution that radiates toward the cell periphery from a perinuclear cage, vimentin in homozygous *GAN* mutant U-2 OS cells organizes into a cluster apposed to one side of the nucleus. Vimentin in these cells is degraded by gigaxonin overexpression, as reported for other cell types. These phenotypes, coupled with factors such as proliferation rate, spread area, species, and amenability to processes such as transfection make these cells a useful model for quantitative high content screening (qHCS). Partial funding provided by Hannah's Hope Fund.

P545**Board Number: B693****NF68, a possible new player in pituitary plasticity.**A. Guardado¹, V. Ovando², T. Fiordeliso¹;¹Departamento de Ecología y Recursos Naturales, Facultad de Ciencias, UNAM, Mexico City, Mexico,²Instituto Nacional de Ciencias Medicas y Nutricion, Mexico City, Mexico

Being the systemic hormonal regulator, the anterior pituitary (AP) is exposed to a complex regulation system; endocrine cells identify and integrate signals from different origins, performing necessary

adjustments in secretion rates and endocrine cell population required to effectively respond to a different physiological condition. Intracellular endocrine cells in the AP respond, among others, with a series of cytoskeletal modifications controlling, hormonal synthesis and release, cell division, and other processes. In non-neuronal cells intermediate filaments intervene in the rearrangement of other cytoskeletal filaments, regulate vesicular transport, and DNA repair. In this work we used Western blotting and double immunofluorescence staining to quantify and study changes in the 68kDa neurofilament (NF68) expression in AP of two months old male and estrous female rats which were adrenalectomized, thyroidectomized, gonadectomized, or lactating (with and without suction stimuli) in order to provoke a physiological demand for certain hormones. Monoclonal mouse anti-NF68 and polyclonal rabbit antibodies for each AP hormone (GH, PRL, ACTH, TSH, FSH, LH) and actin were used for each experiment respectively. All images were analyzed with ImageJ. Our Western blot results show a twofold to threefold increase in total NF68 expression in all experimental condition (with the exception of lactating females without suckling stimuli); two different NF68 isoforms can be seen, one of them in the 68kDa band and the other near the 50kDa band, both sharing a common epitope recognized by our monoclonal antibody. Changes in NF50 and NF68 expression vary depending of experimental condition and sex. Double immunofluorescence staining results show NF68 expression in every endocrine cell population in the AP (with the exception of corticotropes) as well as an increase in NF68 expression in response to experimental condition, represented by an increase in the percentage of double positive cells (NF+/Hormone+). The increased total NF68 expression, NF50 and NF68 patterns of expression, as well as the increased NF+ cell proportions seen in our experimental conditions seem to suggest the presence of functionally different populations of NF+ endocrine cells in the AP. Considering all these evidence, we believe that NF may have a role in the regulation of NF+ cells' response to stimuli, contributing to AP plasticity. We will continue evaluating the possible effects in secretion and mitotic index of these NF+ cells in an attempt to determine if they are indeed functionally different from NF-cells.

P546

Board Number: B694

Nuclear damage in highly constrained migration: from lamina defects to DNA breaks.

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Cells *in vivo* are sometimes required to migrate through tight spaces that are much smaller than the largest organelle, their nucleus. Micro-pore migration of lung cancer cells causes nuclear blebs with segregated lamins as well as DNA tethering and breaks. Nuclear blebs seen in the majority of cells are enriched in lamin-A and deficient in both lamin-B and DNA, but the cells are viable with a normal rate of post-migration proliferation. Phosphorylation of lamin-A, which relates to turnover under low stress,

decreases with migration, while phosphomimetic and progeria mutants of lamin-A exhibit distinct differences. Knockdown of lamin-A induced the frequent formation of DNA tethers that extrude from the main nuclear body through a gap in lamin-B and to the pore that the cell migrated through. Double strand breaks also increased and are consistent with subsequent cell death. The findings reveal a crucial role for the lamins in cell migration and survival, likely through DNA protection.

P547

Board Number: B695

FAM83H regulates organization of the keratin cytoskeleton in colorectal cancer cells.

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During invasion and metastasis, cancer cells lose, at least partially, properties of epithelial cells such as rigid cell-cell and cell-extracellular matrix adhesions. The keratin cytoskeleton is one of the important factors that maintain the epithelial properties. FAM83H, an essential protein for amelogenesis, was recently suggested to be involved in cancer. In this study, we show that FAM83H and casein kinase 1 α (CK-1 α) are novel proteins that modulate organization of the keratin cytoskeleton in colorectal cancer cells. We first performed proteomic analysis to search proteins that can interact with FAM83H, and we identified CK-1 α and keratins. This identification led us to hypothesize that FAM83H and CK-1 α are involved in organization of the keratin cytoskeleton. To investigate the role of FAM83H in organization of the keratin cytoskeleton, we performed experiments using knockdown and overexpression of FAM83H. The overexpression disassembled keratin filaments, whereas the knockdown thickened those, suggesting that the filamentous state of keratins is regulated by FAM83H. Next, to elucidate the involvement of CK-1 α in this FAM83H-governed organization of the keratin cytoskeleton, we used FAM83H mutants with alanine substitutions in their CK-1 α binding sites. These FAM83H mutants could not bind to CK-1 α and thus failed to recruit CK-1 α to keratin filaments. Importantly, these alanine mutants could not reorganize the keratin cytoskeleton, suggesting that CK-1 α binding is required for FAM83H to reorganize the keratin cytoskeleton. The important role of the CK-1 α binding was further supported by the results that FAM83H dominant negative mutants, which possess the ability to bind to CK-1 α but not to keratins, aggregated keratin filaments through sequestering CK-1 α from keratin filaments. In addition, we show that FAM83H is involved in formation of desmosomes, which are maintained by the keratin cytoskeleton. Lastly, we performed immunostaining of human colorectal cancer tissues to explore the role of FAM83H in vivo. Overexpression of FAM83H was observed in colorectal cancer cells that were invading through stromal cells. In fact, FAM83H-overexpressing cancer cells exhibited disassembled keratin filaments and characteristic features indicating loss of epithelial properties. Taken together, these results suggest that overexpression of FAM83H may impair the epithelial properties by disrupting the keratin cytoskeleton during invasion of colorectal cancer cells. (Kuga et al., J. Cell Sci. 2013; 126:4721-4731)

P548**Board Number: B696****The motile properties and distribution of mitochondria are altered in fibroblasts derived from Giant Axonal Neuropathy patients.**J. Lowery¹, N. Jain¹, R.D. Goldman²;¹Cell Molecular Biology, Northwestern Univ, Chicago, IL, ²Department of Cell and Molecular Biology, Northwestern University, Feinberg School of Medicine, Chicago, IL

Giant Axonal Neuropathy (GAN) is a rare inherited neurological disorder caused by mutations in the GAN gene, which encodes gigaxonin, a predicted E3 ligase adapter protein. Neurons and fibroblasts of GAN patients contain aggregates of intermediate filaments (IFs) caused by the improper degradation of IF due to dysfunctional gigaxonin. In GAN patient fibroblasts, mitochondria are abnormally distributed inside and surrounding vimentin IF (VIF) aggregates. Based on this observation, we used live cell imaging and quantitative fluorescence microscopy to compare mitochondrial motility in fibroblasts derived from GAN patients and normal human fibroblasts. By measuring the displacement and instantaneous velocities of individual mitochondria it was determined that mitochondrial motility was significantly reduced and altered in GAN. This reduction in the motility of mitochondria in GAN patient cells appears to be associated with the abnormal tethering of mitochondria to VIF which is also associated with abnormalities in the cytoplasmic distribution, morphology and membrane potential of mitochondria. In an attempt to mimic the disease phenotype, normal fibroblasts were treated with shRNA designed to lower the expression levels of gigaxonin by 75%. This reduction in gigaxonin induces aggregates of VIF which are similar to those seen in GAN patient cells. Mitochondrial motility was also significantly inhibited in these cells. Normal mitochondrial motility can be restored in GAN patient cells by transiently expressing wt gigaxonin. Furthermore, not all organelle motility is inhibited in GAN fibroblasts as shown by the detection of normal lysosome movements in GAN patient cells compared to control cells. Our results demonstrate that both the abnormal motility and the abnormal distribution of mitochondria in GAN patient fibroblasts are due to pathologically significant changes in VIF networks but not to changes in microtubule networks. Supported by NIGMS Diversity Supplement-5P01GM096971-03 and Hannah's Hope Fund.

Directed Cell Migration

P549

Board Number: B697

Directional 3D cell migration is mediated by branching actin protrusions and matrix deformation.

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Anchorage-dependent cells maneuver the extracellular matrix (ECM) by coordinated dynamics of actin and integrin-mediated adhesions that serve as major mechanical and signaling structures. Traditional 2D models have shown that cells on 2D substrates interact with the ECM on the basolateral side, exhibit relatively flat phenotype, establish apical-basal polarity, and migrate freely on a horizontal plane. However, most physiological processes in vivo are in a 3D microenvironment, where cells engage the matrix in all directions, adapt a spindle or stellate morphology, and cleave the matrix via metalloproteinase (MMP) to create physical migration paths. Taken together, the mode of cell migration in 3D, compared to 2D, is highly distinctive and not well-understood. In this study, we utilized an engineered microfluidic platform and high resolution image analysis to investigate how actin, adhesions, and cell mechanic are regulated during 3D chemotaxis.

Primary human endothelial cells were plated to form 3D perfusable endothelium imbedded in collagen, and directional chemotactic invasion and migration were induced by gradients of growth factors. Cellular morphometrics were measured over time to analyze 3D dynamics and structural reorganization. Fluorescent beads in the ECM were tracked over time to obtain the 3D ECM deformation field generated by cells. The role of cellular contractility in 3D migration was determined by measuring changes in the cytoskeleton and cell mechanics after perturbing myosin II.

Cells migrating directionally towards the gradient within 3D ECMs exhibit elongated actin protrusions. These pseudopodia, unlike broad lamellipodia generated by 2D cells, are highly dynamic and can branch out. Adhesions, identified by paxillin, form along the protrusions, and they remain relatively small. No well-delineated structural morphogenesis as observed in 2D cultures occurs in 3D. Quantifications of ECM deformation indicate that migrating cells exert 3D forces all around, including around the cell body and along the pseudopodia. The elongated actin structures become significantly disorganized when myosin-mediated contractility is inhibited. The persistence of pseudopodia is low, compared to lamellipodia, suggesting that multiple protrusions in one direction promotes synergy for migration efficiency. These findings reveal that chemotaxis in 3D matrices involves different and more complex cell mechanics and cytoskeletal organization, consisting of punctate adhesions and branching actin.

P550**Board Number: B800****Targeted Deletion of the Formin FMNL1 Suppresses the Macrophage Inflammatory Response.**M.R. Miller¹, S.D. Blystone²;¹SUNY Upstate Medical University, Syracuse, NY, ²Department of Cell and Developmental Biology, SUNY Upstate Medical University, Syracuse, NY

Migration of macrophages to inflammatory loci or sites of injury mandates successful engagement and penetration of complex connective tissues. Specialized adhesion structures, termed podosomes, utilize integrin receptors in conjunction with signaling, adaptor and cytoskeletal proteins for traction during migration. We previously reported the functional and physical association between macrophage podosomes and FMNL1, a member of the formin family of actin binding proteins. In vitro, FMNL1 can affect actin filament dynamics through nucleation, elongation, bundling and severing. To determine the role of FMNL1 in podosome function and macrophage migration, we generated a murine deletion model system by introducing loxP sites to the FMNL1 allele. Murine macrophages deficient for FMNL1 were purified from progeny of FMNL1 floxed mice bred with mice expressing Cre recombinase via the macrophage specific promoter LysM (FMNL1^{-/-}). In a modified Boyden chamber we observed a 33.5 percent reduction in FMNL1^{-/-} macrophage migration across a porous barrier in comparison to wild type macrophages. FMNL1^{-/-} macrophages exhibit 47.8 percent reduction in cells developing podosomes and a 12.6 percent increase in cell spreading. Importantly, a 38.6 percent reduction in residential macrophages of the liver was observed. An in vivo inflammation model using thioglycollate induced peritonitis shows a 15.2 percent decrease in F4/80 (+) cells, a macrophage specific marker, in FMNL1^{-/-} mice. Interestingly, FMNL1^{-/-} macrophages show elevations of active GTPases including Rac1, CDC42, and RhoA with increases of 31.2, 20.4, and 38.6 percent, respectively, suggesting compensatory mechanisms responding to disrupted cytoskeletal dynamics. We have previously reported that human macrophages with reduced levels of FMNL1 through siRNA targeting exhibit deficient adhesion, migration, and loss of podosome stability. Our data support a model where FMNL1 affects the integrity of podosome structure and function necessary for macrophage immune-related migration events in vivo.

P551**Board Number: B801****Polarity mechanisms such as contact inhibition of locomotion regulate persistent rotational motion of mammalian cells on micropatterns.**B.A. Camley^{1,2}, Y. Zhang^{3,4}, Y. Zhao^{2,5,6}, B. Li^{2,5}, E. Ben-Jacob^{4,7}, H. Levine^{4,8}, W. Rappel^{1,2};¹Physics, University of California, San Diego, San Diego, CA, ²Center for Theoretical Biological Physics, San Diego, CA, ³Physics, Rice University, Houston, TX, ⁴Center for Theoretical Biological Physics, Houston, TX, ⁵Mathematics, University of California, San Diego, San Diego, CA, ⁶Mathematics, George Washington University, Washington DC, DC, ⁷Physics and Astronomy, Tel Aviv University, Tel Aviv, Israel, ⁸Bioengineering, Rice University, Houston, TX

Pairs of endothelial cells on adhesive micropatterns rotate persistently, but pairs of fibroblasts do not; coherent rotation is present in normal mammary acini and kidney cells but absent in cancerous cells. Why? To answer this question, we develop a computational model of pairs of mammalian cells on adhesive micropatterns and study the conditions under which persistent rotational motion (PRM) emerges. Our model couples the shape of the cell, the cell's internal chemical polarity (modeled by a reaction-diffusion process), and physical interactions between cells such as volume exclusion and adhesion. We show that PRM can emerge from this minimal model, and that the cell-cell interface shape may be influenced by the nucleus, which is often neglected in simulations. We study the effect of various mechanisms of coordinating polarity between cells on rotational motion, including contact inhibition of locomotion, neighbor-alignment, and velocity-alignment (where cells align their polarity to their velocity). These polarity mechanisms strongly regulate PRM: small differences in polarity mechanisms can create significant differences in collective rotation. We also show that an individual cell's persistence of direction can regulate PRM: cells with primarily random motility do not develop PRM. We argue that the existence or absence of rotation under confinement may lead to insight into the cell's methods for coordinating collective cell motility.

P552**Board Number: B802****Nanotopography induced guidance of actin polymerization waves and the underlying mechanisms in amoeboid neutrophil migration.**S. Das¹, C. Guven¹, X. Sun², M. Hourwit², J. Fourkas², W. Losert³;¹Dept of Physics and Institute for Physical Science and Technology, University of Maryland, College Park, MD, ²Dept of Chemistry and Biochemistry, University of Maryland, College Park, MD, ³Dept of Physics, University of Maryland, College Park, MD

Directional cell migration due to external chemical gradients is well established, and yet has limitations, especially over long distances. Topography induced contact guidance provides a ubiquitous alternative, for directional migration across long distances. In this study, we show that nanotopography can trigger

directional cell migration, opening up a broad range of possible applications. We found that actin waves are linked to this contact guidance process. Further, we investigate the mechanisms underlying this contact guidance in amoeboid migration using HL-60, neutrophil-like cell line. We find that cells are polarized with guided actin polymerization and cell migration on nanotopography. Tracking of the actin waves using the intensity differences of actin polymerization between successive frames allowed us to measure the distribution of actin-wave speeds and directions relative to the nanopatterned surfaces. Utilizing this technique we show that actin preferentially polymerizes to the nanotopography over a range of spatial periodicities and the velocity of actin polymerization waves is also increased along the nanotopography. Preliminary results show that nanotopography local protrusions spatially distribute the focal-adhesion associated actin regulator paxillin in polarized, migrating cells. We further demonstrate that the contact guidance efficiency, i.e., the degree to which a cell responds to nanotopography, depends on the local details in the nanotopographic features and is connected with the intrinsic actin dynamics in migratory amoeboid cells.

P553

Board Number: B803

Exosomes mediate LTB₄ signal relay during neutrophil chemotaxis.

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The secretion of secondary chemoattractants represents a powerful mechanism by which chemotaxing cells maintain robustness and sensitivity to highly diffusible primary chemoattractant signals. Once secreted, these secondary chemoattractants form a gradient to recruit neighboring cells, thereby dramatically increasing the range of detection. Leukotriene B₄ (LTB₄) has been shown to be such a secondary chemoattractant during neutrophil chemotaxis, although the secretion and the process by which such a small molecule maintains a stable long acting gradient remain to be determined. We hypothesize that in neutrophils, secreted LTB₄ is packaged into extracellular vesicles called exosomes that are periodically released to form a stable gradient. We now show that upon stimulation with the primary chemoattractant fMLP, neutrophils secrete extracellular vesicles that are enriched with the exosomal markers CD63 and HSP70 and contain LTB₄. Importantly, we also find that the enzymes involved in the synthesis of LTB₄, 5-lipoxygenase (5-LO), 5-lipoxygenase-activating protein (FLAP) and LTA₄ hydrolase, are present in purified exosomes, suggesting that active synthesis of LTB₄ occurs in secreted exosomes. Furthermore, we found that 5-LO-mCherry, which primarily associates with the nuclear membrane in resting neutrophils, redistributes into CD63-positive vesicles that coalesce at the back of chemotaxing neutrophils upon activation with fMLP. Exogenous addition of exosomes to resting primary neutrophils leads to a LTB₄ receptor-dependent polarization and migration and a concomitant activation of biochemical pathways known to be involved in migration. Finally, decrease of exosome secretion by inhibiting Rab27a and n-Sphingomyelinase-2 activity leads to reduction of neutrophil recruitment and LTB₄ relay, an effect that recapitulates the pharmacological inhibition of LTB₄ synthesis. Together, these findings strongly suggest that LTB₄ secretion is mediated by exosome release during

neutrophil chemotaxis and implicate this novel delivery mechanism to mediate signal relay in mammalian systems.

P554

Board Number: B804

Mapping of motility behavior in neutrophil-like HL-60 cells using Linear Optimal Transportation (LOT) and dimensionality reduction techniques.

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Motile cells respond to changes in their environments, including temperature variations, changes in matrix structure, or the presence of chemoattractants. The change in motility is typically measured and characterized by changes in cell morphology, speed, shape, trajectory, or directional persistence. While these parameters can be readily obtained from microscopy data, only a subset of these parameters are normally used at the same time to analyze change in motility in a typical experiment, as it becomes increasingly difficult to visualize changes and interpret trends as the dimensionality of the data set increases. On the other hand, in order to investigate molecular mechanisms that underlie cell motility, it is necessary to connect changes in cell morphology, speed, shape, trajectory, and directional persistence to the underlying cytoskeletal dynamics by including data on subcellular structure movements as well as protein interaction and distribution, such as tracking the intensity and localization of nuclear DNA, actin/myosin, and focal adhesion markers over space and time. The resulting data set increases the dimensionality even further, and the complexity in analyzing such a high dimensionality data set also increases. Furthermore, changes in protein interaction and distribution often vary non-linearly over space and time, and traditional linear techniques such as Principal Component Analysis (PCA) may fall short on extracting critical trends in space, time, and frequency directly from these raw image data sets. However, by employing a technique called Linear Optimal Transport (LOT), which combines transportation-based metrics for image distance calculations while providing linear representations of the original data sets, intensity-based images can be transformed and embedded onto Euclidean space. This mapping thus enables the application of linear techniques such as PCA on high-dimensional, non-linear data sets for the study of motility behavior.

P555**Board Number: B805****Two different modes of collective cell movement.**A. Combedazou¹;¹University Paul Sabatier, LBCMCP CNRS UMR 5088, Toulouse, France

Collective cell migration is a central process during embryo morphogenesis, wound repair and cancer invasion. The hallmark of collective migration is that cells need to coordinate their behavior cooperatively in response to guidance signals. This coordinated migratory behavior is mainly referred as a polarized cell movement, in which chemoattractant gradients control the choice of leader cells from followers. However, this concept of leadership during collective chemotaxis is contradictory to a rotation or tumbling movement mainly reported in collective border cell migration during *Drosophila* ovary development. While the polarized non-rotational behavior has been extensively studied, the rotational phase is less understood notably because of a lack of tools to precisely quantify it and discriminate it from the polarized non-rotational phase in a 3D context. Consequently, the controlling mechanism and biological function of collective rotation are still completely unknown. To address these questions, we used *ex vivo* culture and time-lapse live cell imaging to monitor and measure different movements of border cell cluster, in WT and various genetic background. Using a mathematic approach to quantify the rotational behavior, we demonstrated that the cross-talk between the ROCK – Non-muscle MyosinII (NMII) signaling and the polarized activity of small GTPase Rac governs the transition between polarized non-rotational and rotational modes of collective migration. We found that the occurrence of rotation is mainly dependent on the activity of ROCK and NMII in collective cluster. Oppositely, the polarized Rac activity protrusion are correlated with the polarized non-rotational behavior. In order to confirm the switch control of collective polarized non-rotation vs. rotation by this signaling cross-talk, we used the genetic manipulation and an optogenetic tool called photoactivatable-Rac to change the ROCK/NMII activity and the Rac polarity respectively. We demonstrated that the ROCK/NMII activity and the Rac polarity antagonize each other. Thus this negative cross-talk efficiently governs a switch between these two modes of collective movement. Our further work indicated that the ROCK/NMII signaling negatively affects the Rac activity and protrusion formation locally, which thus leads to a global control of the polarized distribution of both Rac activity and protrusion within border cell cluster. In parallel, we confirmed this phenomenon in collective migration of different types of cancer cells, which have various collectively migratory abilities possibly through the adoption of different movement modes.

P556**Board Number: B806****Topography and stiffness of endothelial layers direct intraluminal crawling of T cells.**J. Doh¹, K. Song¹;¹Department of Mechanical Engineering, POSTECH, Pohang, Korea

Immune cells circulating in inflamed blood vessels undergo series of adhesion cascades to leave out of blood vessels to infiltrate into inflamed tissues. It has been shown that leukocytes crawl substantial amount of distances before they undergo transendothelial migration (TEM), but biochemical/biophysical cues directing the crawling of leukocytes have not been clear. While both endothelial cell (EC) junctions and flow can affect crawling direction of leukocytes, experimental settings currently widely used do not allow systematic examination on which factor has predominant roles on determining intraluminal crawling direction. To directly address this problem, we fabricated well-aligned EC layers by culturing ECs on nanogrooved surfaces and applied shear flow either parallel or perpendicular to the direction of EC orientation. Regardless of flow direction, T cells crawled along the EC orientation. Then, we further identified guiding cues on ECs and found out that T cells tend to crawl along the valleys of topographical landscapes of EC layers while avoiding nuclei of ECs. To directly test whether T cell crawling is guided by the topography of EC layers, EC layers were fixed, dried, and replicated with UV-curable resin poly(urethane acrylate) (PUA). T cells on PUA replicas coated with key adhesion molecules for crawling also crawled along the EC orientation, suggesting topography of EC layers is one of the major factors guiding T cell crawling. However, T cells on PUA replicas frequently crossed over nuclei of ECs, meaning that the tendency of avoiding nuclei of ECs by crawling T cells cannot be explained by topography effect. When lamin A/C of ECs were knocked down by siRNA to reduce nucleus stiffness of ECs, crawling T cells no longer avoided nuclei of ECs, meaning T cells sense stiff nuclei of underlying ECs during crawling. By using pharmacological inhibitors for arp 2/3 and cdc42, we found out that T cells utilize lamellipodia to sense topography of EC layers and invasive filopodia to sense stiff nuclei of ECs. Importantly, inhibitor-treated T cells crawled much longer distances than untreated T cells before they underwent TEM, suggesting that lamellipodia- and filopodia-mediated biophysical environments sensing of T cells are critical for optimal intraluminal path finding.

P557**Board Number: B807****Osmotic surveillance mediates rapid wound closure through nucleotide release.**W. Gault¹, B. Enyedi¹, P. Niethammer¹;¹Cell Biology, Sloan-Kettering Institute, New York, NY

While tissue intrinsic wound detection mechanisms exist (e.g. loss of contact inhibition or chemotactic guidance), little is known about the signals induced by an organism's extrinsic environment. Injury to wet epithelia (e.g. gastrointestinal/mucosal surfaces or fish skin) permits entry of environmental liquids

into the tissue and is associated with rapid wound closure. As osmotic cues from the environment promote wound detection by leukocytes in zebrafish, we tested the hypothesis that environmental osmolarity is a mediator of wound detection and closure by epithelial cells. Using time-lapse fluorescence microscopy and intravital luminescence imaging in a zebrafish larval tailfin model of wet epithelial wound closure, we found that an osmotic difference between the interstitial fluid and the external environment drives hypotonicity-induced ATP release, leading to long-range activation of basal epithelial cell migration and rapid wound closure. We determined that the spatiotemporal pattern of tissue velocity during wound closure is controlled by the extracellular nucleotide degrading ectonucleoside triphosphate diphosphohydrolase 3 (Entpd3). Our data supports that the larval zebrafish skin integrates two spatially distinct yet functionally interrelated modes of wound closure: an environmentally (hypotonicity)-induced lamellipodial sheet migration in the basal cell layer, and an environmentally independent purse-string contraction at the suprabasal wound margin. Overall, we define a novel environmental osmotic surveillance circuit, which is integrated with tissue intrinsic mechanisms to promote rapid, wet epithelial wound closure. These results support that extrinsic environmental exposure is an important master stimulus of the wound response for both leukocytes and epithelial cells in vivo.

P558

Board Number: B808

Investigating electrotaxis within the confines of cell clusters.

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Electrical signals arise naturally within the human body in multiple locations including blood vessels, the skin, and ducts. Externally applied electric fields have previously been shown to influence collective cell migration in cases such as wound healing. Additionally, it has been reported that cells within clusters are more sensitive to electric fields than their isolated counterparts. The purpose of this study was to quantify the properties of motion of non-transformed mammary epithelial cells (MCF-10A), isolated and within clusters, under an electric field. To do so, cells were seeded into an electrotactic chamber constructed with 0.15 mm glass coverslips attached to a polystyrene dish with silicon adhesive. Electric current was generated and maintained by an external potentiostat while cell movement was recorded in real time by time lapse microscopy. Highly metastatic breast cancer cells (MDA-MB-231) were confirmed to migrate towards the anode of an electric field, as reported previously. Surprisingly, MCF-10A cells were also found to migrate in a directed fashion towards the anode, with 51.8%, 57.0%, 73.8%, 83.8%, and 89.5% migrating towards the anode at field strengths of 0, .13, .26, .51, and 1.0 V/cm, respectively. The directedness of cell migration was defined as the cosine of the angle between the vector of the path length and a vector parallel to the electric field sharing the same origin. A directedness of 1 therefore indicates a cell moving directly towards the anode, 0 indicates random migration, and -1 indicates direct migration to the cathode. The directedness under these field strengths

were found to be .009, .113, .397, .600, and .705, respectively, indicating a linear dependence on the log of the electric field strength. Likewise, for cell clusters, 53.9%, 70.1%, 84.2%, 95.0%, 99.0% of cells migrated towards the anode under electric fields of 0, .13, .26, .51, and 1.0 V/cm, respectively, with corresponding directedness values of 0.081, 0.332, 0.560, 0.772, and 0.840. This increased sensitivity to the electric field within clusters is attributed to intercellular signaling rather than steric effects alone, as evidenced by analyzing the persistence of motion at these electric fields. Interestingly, isolated cells were found to achieve directedness of motion more quickly than their counterparts within clusters. Although cell-cell interactions afford cells within clusters a higher degree of sensitivity to electric fields, they also were found to dampen the dynamics of the associated electrotactic response.

P559

Board Number: B809

Lipid Rafts Mediate Electric Field-induced Directional Migration.

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Endogenous electric fields arise during development, wound healing, and at epithelial interfaces. Cells respond to the electric fields with changes in directional migration, morphology, and gene expression. As such, these fields are involved in morphogenesis, healing, and metastasis. While the significance of bioelectricity is widely acknowledged, mechanisms behind electrosensing are not well understood. Previous studies proposed that electrophoretic and electroosmotic forces act on the charged molecules on the plasma membrane and result in redistribution of membrane molecules that lead to polarized intracellular signaling. Membrane proteins such as EGF receptors and integrins have been found to exhibit polarized distribution in direct current electric fields. However, due to the size, concentration, and diffusivity of these proteins, they are unlikely to be the primary response element to dynamic electric fields at relative high frequencies. Using alternating and direct current (AC and DC) fields, we demonstrate that lipid rafts are the primary response elements for directional migration in the field. Disruption of lipid raft integrity with M β CD and cholesterol eliminates polarized integrin distribution and cell migration directionality. Furthermore, caveolin-1 knockdown reduced polarized distribution of integrin and RhoA, which mediates migration directionality. Our results establish a new mechanism for cell electrosensing and provide a new role in lipid raft mechanotransduction.

P560**Board Number: B810****On the mechanism of durotaxis in motile cells.**M. Riaz¹, M. Versaevel¹, S. Gabriele¹;¹University of Mons, Mons, Belgium

Many dynamic processes, including cytoskeletal polymerization and cell-substrate interactions, determine morphological (e.g. shape and dimensions of cells) and directional (e.g. speed, direction and persistence) parameters of migrating cells. A large number of studies have characterized the mechanical behavior of stationary cells in response to matrix stiffness changes, however there have been no comprehensive efforts to elucidate the mechanisms by which matrix stiffness determines motile cell behavior. To this end, we have studied the mechanosensitive response of fish keratocytes, which are among the fastest moving animal cells. Based on a large population of cells, our results demonstrate that keratocyte morphological parameters (shape, aspect ratio, lamellipodial curvature, etc.) are significantly influenced by the matrix stiffness over a wide range of rigidities (from kPa to GPa). These observations suggest that matrix stiffness can be a valuable tool for controlling the natural phenotypic variability of keratocytes. Furthermore, migrating parameters (directionality, speed, motility coefficient) were also observed to be controlled by matrix stiffness, suggesting that the substrate rigidity dictates the level of polarization and directionality of motile cells. Surprisingly, motile cells were observed to maintain a constant spreading area during their polarization over seven orders of magnitude in rigidities, in contrary to previous reports on stationary cells. We further demonstrate that the physical linkage between the cytoskeleton and the substrate is significantly affected by the matrix stiffness and is required for cell polarization. On the whole, our results are consistent with a quantitative physical model in which the overall keratocyte behavior (shape, polarization and directionality) emerges from the impact of the matrix stiffness on the formation of focal adhesions that creates a frictional slippage, which, in turn, balances myosin-mediated contractile forces.

P561**Board Number: B811****Optogenetic control of chemokine receptor signal and T cell migration.**Y. Xu¹, M. Kim¹, Y. hyun¹;¹University of Rochester Medical Center, Rochester, NY

Adoptive cell transfer (ACT) of ex vivo generated immune-promoting or tolerogenic T cells to either enhance immunity or promote tolerance in patients has been used with some success. However, effective trafficking of the transferred cells to the target tissue sites is the main barrier to achieving successful clinical outcomes. Here, we developed a strategy for optically controlling T cell trafficking using a photoactivatable (PA) chemokine receptor. PA-CXCR4 transmitted intracellular CXCR4 signals in response to 505-nm light. Localized activation of PA-CXCR4 induced T cell polarization and directional migration ("phototaxis") both in vitro and in vivo. Directing light onto the melanoma was sufficient to

recruit PA-CXCR4-expressing tumor-targeting cytotoxic T cells and improved the efficacy of adoptive T cell transfer immunotherapy, with a significant reduction in tumor growth in mice. These findings suggest that the use of photoactivatable chemokine receptors allows remotely controlled leukocyte trafficking with outstanding spatial resolution in tissues and may be feasible in other cell transfer therapies.

P562

Board Number: B812

Using a novel microfluidic device to define the role of TRIM9 in Netrin-based axon guidance.

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The functional network of neurons in the brain is established during development when extending axons are guided by extracellular guidance cues toward postsynaptic partners. One such cue, Netrin promotes axon branching and attractive axon guidance through its receptor DCC. We recently identified an interaction between DCC and the E3 ubiquitin ligase TRIM9. Using primary cortical neurons from *TRIM9*^{-/-} mice we found that TRIM9 is a novel regulator of Netrin-dependent exocytosis and axon branching in primary cortical neurons. Based on the well-established role of Netrin and DCC in axon guidance, we hypothesized that TRIM9 may be a novel regulator of axon guidance in response to Netrin. In support of this, cortical explants from wildtype mice grow preferentially toward an asymmetric Netrin source, however *TRIM9*^{-/-} explants fail to do so. Axon guidance has typically been investigated using low throughput needle-based assays to establish local gradients of axon guidance cues at the growth cone. Although these assays are useful, they are highly variable, time consuming, and difficult to analyze. We present here a novel PDMS-based microfluidic device in which we can establish a stable, long-lived gradient of Netrin specifically across aligned axons, without exposing neuronal cell bodies. This device is amenable to high resolution, live cell microscopy, long term neuronal viability, and because axons are aligned prior to exposure to Netrin, it also facilitates data analysis. With this device we observe that in wildtype neurons there is robust axon turning toward low concentrations of Netrin. Analysis of *TRIM9*^{-/-} axon turning is ongoing. Defects in outgrowth toward Netrin in vitro are associated with projection defects in vivo in *TRIM9*^{-/-} mice, and an overt spatial learning and memory deficit. We anticipate these defects will be associated with disrupted axon guidance in vitro as well. In conclusion we have identified a novel regulator of Netrin-dependent axon guidance and are using innovative new technologies to define changes in axon guidance in vitro.

P563**Board Number: B813****Collective Epithelial Cell Sheet Adhesion and Migration on Polyelectrolyte Multilayers with Uniform and Gradients of Compliance.**J.S. Martinez¹, T.C. Keller¹, J.B. Schlenoff²;¹Department of Biology, Florida State University, Tallahassee, FL, ²Chemistry, Florida State University, Tallahassee, FL

Polyelectrolyte multilayers (PEMUs) are tunable thin films, which could serve as coatings for biomedical implants. PEMUs built layer by layer with the polyanion PAA (poly(acrylic acid)) modified with a photosensitive 4-(2-Hydroxyethoxy) benzophenone (PAABp) and the polycation PAH (poly(allylamine hydrochloride)) are mechanically tunable by UV irradiation, which forms covalent bonds between the layers, increasing PEMU stiffness. Our study utilized uniformly charged PAH-terminated PEMUs (PAH-PEMUs) that were uncrosslinked, uniformly UV-crosslinked, or UV-crosslinked through a neutral density optical gradient filter forming a continuous compliance gradient to investigate how differences in PEMU stiffness affect the adhesion and migration of epithelial cell sheets underlying scales of the fish *Poecilia sphenops* (Black Molly) and *Carassius auratus* (Comet Goldfish). PAH-PEMUs containing a compliance gradient promoted durotaxis of the cell sheets but not of individual keratocytes. During the progressive collective cell migration, the leader cells in the sheets on the softer uncrosslinked PEMUs and less crosslinked regions of the gradient formed more robust stress fibers and vinculin-containing adherens junctions and focal adhesions, indicating higher levels of intracellular tension, than in the sheets on the stiffer PEMUs or glass. During sheet extension, the ratios of motility velocities between the leader cells and follower cells in the migrating sheets were two times greater on the softer PEMUs than on the stiffer PEMUs or on glass. On the softer, but not on the stiffer PEMUs, the region of leader cells in the sheets exhibited periods of retraction, during which the leader cells lost adhesion to the substrate and retracted toward the more adherent follower cell region. These retraction events were inhibited by blebbistatin (a myosin II inhibitor), which reduced the motility velocity ratios to those measured on the stiffer PEMUs and promoted loss of leader cell-cell connections. Blebbistatin also caused Myosin II disassembly of stress fibers, reorganization of focal adhesions, and increased cell spreading at the leading edge in epithelial cell sheets on glass. Interestingly, cells throughout the interior region of the sheets on uncrosslinked PEMUs conserved their actin and vinculin organization at adherens junctions. Like Blebbistatin, a Rho-kinase (ROCK) inhibitor promoted loss of cell-cell connections between leader cells, whereas a Rac-1 inhibitor altered the lamellipodial protrusion and ruffling patterns in both cell sheet leader cells and individual keratocytes. Investigating how PEMU surface compliance modulates cell sheet adhesion and motility will further our understanding of collective cell migration and durotaxis and suggest potential applications for PEMUs.

P564**Board Number: B814****A BioMEMS Device for Localized Manipulation of the Tumor Microenvironment.**L. Butt¹, J. Sanders¹, A. Clark¹, J.K. Williams¹, M.R. Padgen¹, P.J. Keely², J.S. Condeelis³, J.A. Aguirre-Ghiso^{4,5}, J. Castracane¹;¹SUNY College of Nanoscale Science and Engineering, Albany, NY, ²University of Wisconsin-Madison, Madison, WI, ³Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY, ⁴Black Family Stem Cell Institute, Manhattan, NY, ⁵Division of Hematology and Oncology, Mount Sinai School of Medicine, Department of Medicine, NY

The tumor microenvironment is a complex system which is not fully understood. New technologies are needed to provide a better understanding of the role of the tumor microenvironment in promoting metastasis. The Nano Intravital Device, or NANIVID, has been developed as an optically transparent, implantable tool to study the tumor microenvironment. Two etched glass substrates are sealed using a thin polymer membrane to create a reservoir with a single outlet. This reservoir is loaded with a custom hydrogel blend that contains selected factors for delivery to the tumor microenvironment. When the device is implanted in the tumor, the hydrogel swells and releases these entrapped molecules, forming a sustained concentration gradient.

In conjunction with the NANIVID technology, multiphoton-based intravital imaging has been used to perform in vivo migration experiments utilizing MDA-MB-231 cell-derived mammary tumors in SCID mice, with epidermal growth factor as a chemoattractant. Additionally, the induction of microenvironments implicated in metastasis including hypoxia and altered extracellular matrix composition have been explored using the NANIVID. Induction devices, or iNANIVIDs, were implanted into HEP3 tumors grown on the chorioallantoic membrane of chicken eggs to demonstrate the extent of the influence of the released chemicals. L-ascorbic acid released by the iNANIVID stimulated localized collagen deposition by hTERT fibroblasts. These projects demonstrate the flexibility of the NANIVID to be used as a tool for modifying and investigating tumor microenvironments in a localized and controlled manner.

P565**Board Number: B815****Control of Wnt5a signaling and cell polarity through organelle dynamics.**M. Connacher¹, N. Ahn¹;¹Biochemistry, University of Colorado Boulder, Boulder, CO

Wnt5a plays an emerging role in cancer malignancy and strongly enhances cell invasion in melanoma. We discovered a new intracellular structure, named the “Wnt5a-receptor-actomyosin polarity (WRAMP) structure”, which provides a novel mechanism to link Wnt5a, a signaling ligand that controls cell polarity, to rear-directed signaling and directional cell movement. This structure involves the formation of signaling endosomes, which interact dynamically with F-actin at the cell posterior to create the

WRAMP structure. Formation of the WRAMP structure induces a localized increase in cytoplasmic Ca²⁺, followed by membrane retraction and forward translocation of the cell body. Thus, the WRAMP structure explains how cells can integrate cell polarity with the mobilization of Ca²⁺ and subsequent membrane contractility, key steps needed for cell movement and invasion. It also explains how Wnt5a promotes Ca²⁺ signaling, observed years ago, but never explained mechanistically.

MCAM, a cell adhesion molecule, undergoes endosome internalization in response to Wnt5a and is our key marker for the WRAMP structure in melanoma cells. We have shown that the signaling endosomes contain both MCAM and Fzd3, a Wnt5a receptor Wnt5a. A new mass spectrometry approach, "Dynamic Organelle Trafficking by Signaling (DOTS-MS)" was used to identify other proteins that associate with the endosomes in the WRAMP structure. Several of these proteins were tested by immunofluorescence and confirmed to localize to the WRAMP structure.

The WRAMP structure links to the plasma membrane in a rear-polarized manner through the phosphoinositol binding protein, ezrin, and PI4,5P2. Ezrin, and another related protein moesin, are both required for the formation of the WRAMP structure and an enrichment of PI4,5P2 is found at the tail of the cell near the WRAMP structure in response to Wnt5a. In addition, rear polarity of the WRAMP structure requires the chemokine, CXCL12, which we suggest is responsible for setting up the rear polarity of PI lipids at the plasma membrane, while ezrin binds these lipids and forms a tether for the WRAMP structure.

A key function of the WRAMP structure is to elevate cytoplasmic Ca²⁺ in a highly localized manner, which in turn triggers actomyosin contractility. We have shown that the endoplasmic reticulum-bound Ca²⁺ sensor, STIM1, and the plasma membrane Ca²⁺ channel, ORAI1, localize near the WRAMP structure in response to Wnt5a and cortical ER is present at the WRAMP structure. We suggest that the highly localized Ca²⁺ elevation at the tail of the cells following WRAMP structure formation involves STIM-mediated opening of ORAI channels and demonstrates a potential mechanism beyond their conventional regulation of store-operated Ca²⁺ entry in response to Ca²⁺ depletion.

P566

Board Number: B816

P-selectin Deletion Attenuates Immune Responses to Fat Overload in Visceral Fat Depots.

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Infiltration of adipose tissue by immune cells is associated with insulin resistance in overweight and obesity. Studies have shown that deletion of the leukocyte-expressed P-selectin glycoprotein ligand-1 (PSGL-1) preserves insulin sensitivity in high-fat fed mice. PSGL-1 regulates trans-endothelial extravasation of leukocytes by binding endothelial expressed P- and E- selectin. Interestingly, recent

studies demonstrate that in fat fed mice infiltration of circulating neutrophils into visceral fat depots occurs very early, with peak values reached at 72 hours after consumption of high fat meals. Accordingly, we hypothesized that deletion of P-selectin, the early responder in endothelial inflammation, attenuates neutrophil invasion of the visceral fat following consumption of obesogenic high-fat meals. P-sel+/+ and P-sel-/- C57BL/6 mice were given either a low-fat (10% fat, 70% carbohydrate, 20% protein) or a high-fat (60% fat, 20% carbohydrate, 20% protein) meal by gavage. All groups of mice were then studied at 1, 2, 3, and 4 hours post gavage by intravital microscopy to measure postprandial kinetics of leukocyte rolling and leukocyte adhesion in the microcirculation of the visceral fat of the mesentery. Cell surface expression of P-selectin was measured by immunofluorescence techniques. The number of infiltrated leukocyte populations in visceral and subcutaneous fat depots was measured by FACS analysis. In wild-type mice, administration of high fat meals acutely increased leukocyte rolling and adhesion in the visceral fat microcirculation (p

P567

Board Number: B817

Nuclear deformation and its consequences on cell function during cell migration through 3-D confining environments.

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Cell migration and motility play a critical role in numerous physiological and pathological processes, ranging from development and wound healing to the invasion and metastasis of cancer cells. It is now becoming increasingly apparent that cell migration in 3-D environments imposes additional challenges and constraints on cells, which can have significant impact on cell migration. The deformation of the nucleus, the largest and stiffest organelle in most cells, is thought to be a limiting factor in cell migration through tight spaces smaller than the nuclear diameter. Reduced nuclear stiffness, as seen in lamin-deficient cells, may thus facilitate 3-D cell migration. Furthermore, lamins A and C, the major contributors to nuclear stiffness, are abnormally expressed in many tumors. Here we investigate the effect of lamin A/C expression on 3-D migration, as well as the consequences of nuclear deformation during the migration through tight spaces.

Since current methods to study the migration of cells confined in three dimensions (3-D) are limited by their physiological relevance and compatibility with available imaging techniques, we developed a novel polydimethylsiloxane (PDMS) microfluidic device that allows observation of cell migration along stable chemotactic gradients through precisely defined 3-D constriction channels. Using constrictions as small as 1 μm wide and 3 to 5 μm tall, we visualized the dynamic deformation of nuclei in cells migrating through the constrictions. We observed that the nuclear lamina deforms and buckles during migration through the smallest constrictions. While wild-type cells were substantially slower to migrate through 2 \times 5 μm^2 wide constrictions compared to 15 \times 5 μm^2 large channels, cells with reduced levels of lamins A and C displayed less or no slowdown in the narrow constrictions, demonstrating the important role of

nuclear stiffness in 3-D cell migration. To understand whether cells can adapt their nuclear or cytoskeletal organization to facilitate migration through narrow constrictions, we are currently studying the ability of cells to migrate through multiple constrictions in series. In addition, we are investigating whether cytoskeletal forces and nuclear deformation may induce nuclear rupture and DNA damage in cells migrating through narrow constrictions.

A better understanding of how cells overcome the mechanical confinement of their surroundings is highly relevant for a variety of physiological phenomena. The migration of cells through dense tissues is a hallmark of metastatic cancer spreading and understanding the factors that limit 3-D migration, as well as the functional consequences of cell deformation, will lead to avenues for cancer therapies.

P568

Board Number: B818

The Role of Chemokines in Collective Cell Migration of Zebrafish Keratocytes.

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The role of chemokines in explant zebrafish keratocytes is of interest for collective cell migration in epithelial wound healing models. Several studies have been conducted examining the specific role of chemokines in processes involving collective movement of epithelial cells. Stromal cell-derived factor-1 (CXCL12) is a chemoattractant that has been shown to activate various signaling pathways stimulating inflammation, homeostatic responses, angiogenesis and metastasis. CXCL11 is a chemokine acknowledged to have similar roles, though it has not been as extensively studied. To date, the role of these chemokines has not been examined in zebrafish keratocyte motility. Therefore, our focus was to determine the role of CXCL11 and CXCL12 in the collective migration of zebrafish keratocytes. The addition of exogenous CXCL11 to zebrafish explant cultures resulted in a decreased rate of migration, changes in cell morphology, accelerated expression of N-cadherin and disassociation of beta-catenin from E-cadherin over time when compared with untreated keratocytes. In contrast, the addition of exogenous CXCL12 resulted in an increased rate of migration when compared with CXCL11 and untreated keratocytes. CXCL12 treatment showed similar changes in cell morphology and N-cadherin expression as with CXCL11 treatment, but at later time points. We also analyzed changes in several biomarkers of EMT over time in chemokine-treated vs. untreated cells. Together, our results suggest that both CXCL11 and CXCL12 are playing significant, but likely different, roles in the collective cell migration of zebrafish keratocytes, and support the hypothesis that these chemokines are promoting an epithelial to mesenchymal transition (EMT) process.

P569**Board Number: B819****Production of Red Fluorescent CXCL12 Overexpressing Mesenchymal Stem Cells (MSC).**M. Reis¹, M.U. Maldonado², R.F. Stout Jr.³, L. Zamproni¹, R. Sessa⁴, P. Matsumoto⁴, S. Han⁴, D.C. Spray³, M. Porcionatto¹;¹Bioquímica, UNIFESP, São Paulo, Brazil, ²Dominick P Purpura, Albert Einstein College of Medicine, Bronx, NY, ³Dominic P. Purpura Department of Neuroscience, Albert Einstein College of Medicine, Bronx, NY, ⁴Biofísica, UNIFESP, São Paulo, Brazil

Neural stem/progenitor cells (NSC) migrate towards a CXCL12 gradient following activation of its receptor (CXCR4). In response to a brain injury, reactive astrocytes express and secrete the chemokine CXCL12, forming a gradient that attracts NSC from the subventricular zone. Our proposal is to generate red fluorescent CXCL12 overexpressing mesenchymal stem cells (MSC) to be used in mouse models for traumatic brain injury (TBI) and stroke. For this aim we constructed a lentiviral vector encoding a chimera formed by CXCL12 and mKate fluorochrome (CXCL12-mKate). The vector was transfected into Hek293 cells and the amount of chimeric CXCL12 secreted to the medium was quantified by ELISA. The concentration of CXCL12 in the conditioned medium of cells transfected with vector encoding CXCL12-mKate raised by 20X. In a transwell migration assay, Jurkat cells (CXCR4+) responded to the CXCL12-mKate chimera gradient increasing the number of cells in the lower chamber by 4.64X. Following the observation that CXCL12-mKate produced is biologically active, we produced viral particles using CXCL12-mKate and infected mouse bone marrow derived MSC, and selected infected cells with blasticidin, resulting in a population of 100% positive MSC for CXCL12-mKate. These cells will now be transplanted in a stroke mouse model, produced by electrocoagulation. In conclusion, we describe the production of mouse bone marrow derived MSC that express red fluorescent CXCL12 that can be used to study CXCL12 gradient formation by MSC, as well as, the dynamics of *in vivo* production and secretion of this chemokine.

P570**Board Number: B820****Cell Ratchets.**D. Riveline¹;¹Laboratory of Cell Physics ISIS/IGBMC, Strasbourg, France

Many physiological phenomena involve directional cell migration. It is usually attributed to chemical gradients *in vivo*. Recently, other cues have been shown to guide cells *in vitro*, including stiffness/adhesion gradients or micropatterned adhesive motifs. However, the cellular mechanism leading to these biased migrations remains unknown, and, often, even the direction of motion is unpredictable. In this study, we show the key role of fluctuating protrusions on ratchet-like structures in driving NIH3T3 cell migration. We identified the concept of efficient protrusion and an associated

direction index. Our analysis of the protrusion statistics facilitated the quantitative prediction of cell trajectories in all investigated conditions. We also varied the external cues by changing the adhesive patterns and topographical patterns. Our measurements for the motions are satisfactorily compared to a persistent random walk model. We show that the cell nucleus contributes to the strength of the trap, while cell protrusions guided by the adhesive gradients add a constant tunable bias to the direction of cell motion.

- Caballero, D., R. Voituriez, and D. Riveline. 2014. Protrusion fluctuations direct cell motion. *Biophys J.* 107:34-42.
- J. Comelles, D. Caballero, R. Voituriez, V. Hortigüela, V. Wollrab, A.L. Godeau, J. Samitier, E. Martínez and D. Riveline. 2014. Protrusion fluctuations direct cell motion. To appear in *Biophys J.*

P571

Board Number: B821

Persistent activation of signal transduction networks induces a novel mechanism of cell death.

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The directed migration of cells in response to chemical cues, known as chemotaxis, plays a critical role in normal physiology and disease pathogenesis. Knowledge of the molecular mechanism of chemotaxis is critical to our understanding of the process of metastasis and for developing new therapies to prevent it.

During chemotaxis ‘front’ proteins are recruited to extending pseudopods, which themselves are concentrated at the cell’s anterior. Conversely, ‘back’ proteins dissociate from nascent pseudopods. The Ras/TorC2 pathway has recently been shown to be important for chemotaxis in *Dictyostelium*. A similar conserved mTORC2 pathway has since been shown to play a role in neutrophil chemotaxis. The discovery of the importance of this pathway in chemotaxis challenges the conventional linear, PIP3-centric view of chemotaxis, in favor of a model involving a complex network of parallel pathways connected via feedback mechanisms. In an attempt to decipher the mechanism of chemotaxis we generated pairwise mutations activating front protein RasC, and inactivating back-protein PTEN. Here we report that this combination of RasC and PTEN perturbations trapped cells in a globally activated or ‘front-state’. Front-state “clamped” cells in *Dictyostelium* were extremely spread and flattened. Most surprising was the susceptibility of these altered cells to death by cytoplasmic fragmentation. Front state clamped cells displayed global elevations of front protein signaling at the cortex. Levels of actin-binding probe GFP LimE at the cortex of front-state cells were elevated, as they were at the peak of the chemotactic response at the tips of pseudopods in control cells. Activating Ras signaling whilst simultaneously inactivating PTEN signaling in mammalian cells led to cell spreading and cell death. These results suggest the mechanism of cell death observed in RasC/pten⁻ *dictyostelium* cells is conserved in mammalian cells.

P572**Board Number: B822****The direction of migration of T-lymphocytes under flow depends upon which adhesion receptors are engaged.**G.A. Dominguez¹, N.R. Anderson², D.A. Hammer^{1,2};¹Department of Bioengineering, University of Pennsylvania, Philadelphia, PA, ²Department of Chemical and Biomolecular Engineering, University of Pennsylvania, Philadelphia, PA

T-lymphocyte migration is important for homing, cell trafficking, and immune surveillance. T-lymphocytes express lymphocyte function-associated antigen-1 (LFA-1) and very late antigen-4 (VLA-4), which bind to their cognate ligands, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). These adhesive interactions provide T-lymphocytes with the ability to withstand hemodynamic shear forces to facilitate adhesion and migration along the blood endothelium. This study investigated whether the identity of the receptor and the magnitude of its engagement affects the direction of T-lymphocyte migration under flow. Microcontact printed ICAM-1 and VCAM-1 PDMS surfaces allowed for control of the density, type of adhesion molecule, and blocking of non-specific adhesion. Using a laminar flow chamber, this study demonstrates that T-lymphocytes migrate either upstream or downstream dependent upon ligand type, ligand concentration, and shear rate. T-lymphocytes migrated upstream on ICAM-1 but downstream on VCAM-1 surfaces. This directed migration was also dependent on the type and concentration of ligand. At high shear stresses (8 dyne/cm²), T-lymphocytes favor upstream migration when any ICAM-1 is present, even in the presence of substantial VCAM-1. These results indicate that T-lymphocytes exhibit two different modes of motility depending on ligand composition and the shear rate.

P573**Board Number: B823****Mathematical modeling and data analysis strategies for tandem fluorescent protein timer microscopy screens.**J. Barry¹, A. Khmelinskii², E. Dona¹, B. Fischer³, D. Gilmour¹, M. Knop², W. Huber¹;¹EMBL, Heidelberg, Germany, ²ZMBH, Heidelberg, Germany, ³DKFZ, Heidelberg, Germany

Tandem fluorescent protein timers (tFTs) are optical probes that report on protein abundance and age in living cells. Each probe consists of a pair of fluorescent proteins that have different maturation kinetics. By comparing the intensities of fast- and slow-maturing fluorophores a quantitative measure of protein age can be attained; under steady-state assumptions a single ratiometric microscopy image is sufficient. The tFT approach presents many opportunities for multiple areas of biological research, including high-throughput microscopy and developmental biology. With these opportunities also come new challenges for data analysis. Here we present examples of data analysis methods used to analyse

high-throughput plate reader experiments of a proteome-wide library of yeast strains tagged with the protein timer, which are subject to different environments or genetic perturbations. We present a method for correcting for background autofluorescence that may skew ratiometric readouts for lowly abundant proteins, and also strategies for correcting for plate-to-plate variability that is technical in origin. As a second application, we demonstrate the efficacy of tFTs for quantifying receptor signaling activity, here in the case of the migrating posterior lateral line primordium in zebrafish. We use a mathematical model of time-dependent fluorophore maturation to characterize potential pitfalls for users of the timer, and show how it may be used to optimize experimental design, including the selection of the fluorophore pair in order to maximize the dynamic range of the readout for a given system.

Cell–Cell Junctions 1

P574

Board Number: B825

A functional proteomic study identifies multiple novel regulators of epithelial cell-cell adhesion.

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Although cell-cell adhesion is central to morphogenesis and maintenance of epithelial cell state, our understanding of the complex networks that regulate cell-cell adhesion is significantly less complete than that of cell-matrix adhesion. We previously identified a subset of 26 genes whose perturbation disrupts cell-cell adhesion as part of a large-scale siRNA screen for regulators of collective cell migration. In the current study, we undertook a proteomics approach to identify high-confidence protein interaction networks for the 26 potential cell-cell adhesion promoting proteins (CCAPPs) encoded by these genes. Specifically, we expressed tagged versions of the CCAPPs in MCF10A cells to serve as ‘baits’ in individual immunoprecipitation (IP)/mass spectrometry experiments, and then used a comparative proteomics analysis software suite (ComPASS) to assign confidence scores to the interacting proteins. The resulting networks (containing 432 high-confidence protein interactions) served as a ‘road-map’ linking novel CCAPPs to known adhesion proteins, connecting CCAPPs to each other, and identifying novel interactors for the canonical adherens junction proteins, α - and β -catenin. Specifically, 18 of the 26 CCAPPs co-precipitated with the α - and β -catenin adhesion complex or with other CCAPPs, and another 7 CCAPPs could be connected through interaction with one or more of the 28 shared interacting proteins (SIPs) amongst the baits within our networks. Novel SIPs for α - and/or β -catenin (DUSP23, ERBB2IP, NEK8, ARVCF, CDH4, PKP4, PLEKHA6) were further validated by immunofluorescence and co-IP studies, and the phosphatase DUSP23 was found to support cell-cell adhesion by promoting dephosphorylation of β -catenin at position Tyr 142 to enhance the interaction between α - and β -

catenin. An additional siRNA-based functional analysis of the 28 SIPs is underway to determine whether more of these proteins play a role in promoting cell-cell adhesion; preliminary data has pinpointed a role for one of the SIPs, PPP6C, in promoting strong cell-cell adhesion. Our study characterizes a network of interconnected, functionally validated cell-cell adhesion regulatory proteins as a resource for further investigations of this important process.

P575

Board Number: B826

Remodeling of cell-cell junctions during cytokinesis.

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Cell-cell junctions are required for maintenance of mechanical integrity and barrier function in epithelial tissues. During cytokinesis, the dividing cell and its neighbors undergo major changes in shape and tension as the contractile ring pinches the cell in two. Junctional integrity and barrier function are thought to be preserved even during cell division. However, our understanding of how junctions are maintained and remodeled during cytokinesis in epithelial cells is lacking. In this study, we investigated the behaviors of three types of cell-cell junctions, tight junctions (TJs), adherens junctions (AJs), and tricellular tight junctions (tTJs), during cytokinesis in the *Xenopus* gastrula by immunofluorescence microscopy and live-imaging of fluorescently-tagged junction proteins. Using a small fluorescent tracer molecule, we demonstrated that the barrier function of TJs remained intact throughout cell division. We found that both TJs and AJs invaginated together with the cleavage furrow; this is in contrast to reports in the *Drosophila* epithelium indicating that AJs are locally disengaged at the division site. Notably, the invagination of AJs preceded that of TJs. Furthermore, we observed that two nascent tTJs were formed at the end of cytokinesis, one on each side of the midbody. LSR/angulin-1, a protein unique to tTJs, was recruited to the newly formed tTJ first and was quickly followed by a second tTJ component, tricellulin. Our data provide the first in-depth characterization of dynamic cell-cell junction remodeling during vertebrate cytokinesis and show for the first time how nascent tTJs are formed following cell division.

P576

Board Number: B827

Critical roles for the apical extracellular matrix and EGF-Ras signaling in building, maintaining and remodeling tiny tubes in *C. elegans*.

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Unicellular tubes are formed from single cells that create an internal lumen. Such tubes are found in the microvasculature of mammals and in simple tubular organs of invertebrates. We use the *C. elegans* renal-like excretory organ as a model system to study unicellular tube development. The excretory

organ is composed of three tandemly-connected unicellular tubes, the canal, duct, and pore, which form through de novo epithelialization coupled either to hollowing or wrapping modes of single-cell tubulogenesis. Through forward genetic screening, we found several families of apical transmembrane or secreted proteins (including leucine-rich repeat proteins, zona pellucida domain proteins and lipocalins) that are required for lumen integrity and junction maintenance. Our results point to a critical role for the apical extracellular matrix in maintaining the integrity of narrow bore tubes.

We also showed that EGF-Ras signaling plays multiple roles in building and remodeling the excretory organ; one important role is to permit G1 pore tube delamination. During normal development, the initial pore cell, G1, delaminates from the organ to become a neuroblast and is replaced by a new cell, G2. G1 delamination and G2 intercalation involve cytoskeletal remodeling, interconversion of autocellular and intercellular junctions and migration over a luminal matrix, followed by G1 junction loss. EGF-Ras signaling acts cell non-autonomously in the duct cell to permit G1 junction loss and delamination. In humans, aberrant EGF-Ras signaling promotes tumorigenesis and metastasis. Our results demonstrate that Ras signaling in cells that remain in an epithelium can create a microenvironment that is permissive for neighboring cells to detach.

P577

Board Number: B828

Repositioning and restructuring of adherens junctions in response to mechanical tensions.

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Although adherens junctions are traditionally thought to maintain tissue integrity by passively resisting disruptive forces, they have increasingly been recognized to actively respond to mechanical cues in cell culture systems. This study uses ventral mesodermal cells of gastrulating *Drosophila* embryos as an *in vivo* system to explore the role of mechanical force in re-positioning, remodeling and maintenance of adherens junctions. Using live imaging and quantitative image analysis we tracked individual junction clusters during gastrulation and found that they persist and move apically while being restructured to enhance intensity and density. Importantly the changes in intensity and position are temporally correlated with pulsed myosin activity both at the level of a whole tissue and at the resolution of individual junctional clusters. Consistent with the correlation study, loss of function analysis indicates myosin is required for the remodeling of adherens junctions. Genetic analyses suggest that tension generated by the apical myosin contraction temporarily protects adherens junctions from a Snail-dependent disassembly mechanism that ultimately occurs when the mesoderm has completed its invagination and begin an epithelial mesenchymal transition. Dual-color live imaging shows that changes of junctional clusters in response to tension precede that of the polarity protein Par3/Bazooka. Our results present *in vivo* evidence that adherens junctions are repositioned and restructured in response to mechanical force and polarity proteins follow the change. We also reveal an unexpected role of Snail

in disassembling adherens junctions at post-transcriptional level which can be suppressed by tension-dependent mechanism.

P578

Board Number: B829

Compartmentalized 3D colocalization analysis – a method to quantify epithelial adherens junction assembly.

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Epithelial adherens junctions are multi-protein complexes composed of the transmembrane adhesion molecule – E-cadherin, which in turn binds to the plaque/adaptor proteins – p120-catenin, β -catenin and a-catenin. This complex is further anchored to the actin cytoskeleton. It has been recently shown that for de novo assembly of adherens junctions, the localization and translation of β -actin mRNA to initial cell-cell contacts is required. In the current work we describe a Pearson's Correlation-based method to measure adherens junction assembly. We reasoned since the adherens junction complex is anchored to the actin cytoskeleton, there must be an increase in the spatial correlation of the adherens junction proteins (E-cadherin, p120-catenin, β -catenin, and a-catenin) and the actin cytoskeleton at the cell periphery during junction assembly. Using the calcium switch approach to initiate de novo junction assembly in MDCK cells, we found the indirect immunofluorescence signals detecting the adherens junction proteins and fluorescent Phalloidin signal detecting F-actin were indeed correlated at the cell periphery. In order to track junction assembly with respect to time, we hypothesized that the correlation of these signals in the cytoplasm of the cell would decrease as the junctions are assembled owing to recruitment of the components of the complex. Setting the lower bound of correlation to 0.1 and carrying out a log transformation of the ratio of correlation at the cell periphery to that in the cytoplasm yielded a metric – Peripheral Asymmetric Index (PAI) – which faithfully tracked assembly of adherens junctions across time. RhoA activation is required for the localization of β -actin mRNA which led us to investigate if RhoA signal at the cell periphery correlated temporally with adherens junction assembly. We show that using a RhoA FRET biosensor, RhoA activation at the cell periphery followed similar temporal scales as adherens junction assembly. We then applied this metric to adherens junction assembly with accumulation of active Src protein at the cell periphery. Since activation of Src at the periphery is required for the translation of β -actin mRNA and hence adherens junction assembly, we hypothesized that recruitment of active Src will spatially and temporally correlate with junction assembly. Indirect immunofluorescence for active Src revealed its accumulation at cell periphery as cells assembled adherens junctions. Upon further quantifying PAI for junction assembly we found cell periphery accumulation of active Src and adherens junction assembly were in fact correlated in both space and time. We thus show a new method based on spatial correlation of immunofluorescence signals to quantify adherens junction assembly across time.

P579**Board Number: B830** **α -Catenin coordinates epithelial migration and tissue morphogenesis through a dual-kinase/phosphorylation mechanism.**

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The cadherin-catenin adhesion complex is a key contributor to epithelial tissue stability, mechanotransduction, and dynamic cell movements during animal development and tissue renewal. How cadherins and catenins are regulated to accomplish these functions is not fully understood. Here we examined the role of phosphorylation of α -catenin (α -cat), a multi-domain scaffolding protein that links the cadherin/ β -catenin complex to the actin cytoskeleton and is a mechano-sensor for the cadherin/catenin complex. Phospho-proteomic screens and our mass-spectrometry analysis identified several phosphorylation sites in a flexible linker located between the central M-region and the C-terminal actin-binding domain of mammalian α -E-cat and *Drosophila* α -cat. Our data show that this 'P-linker' is phosphorylated hierarchically by casein kinase (CK) 2 and 1 and comprises the main phosphorylated region in α -E-cat. Assessment of α -cat phosphorylation in *Drosophila* and mammalian cells indicate that the P-linker and its phosphorylation and dephosphorylation are required for normal cadherin-catenin complex function. Although P-linker mutations did not interfere with cadherin-catenin complex assembly and epithelial integrity in flies, fewer embryos reached adulthood indicating a required role during later developmental stages. In mammalian cells, phosphorylation within the P-linker is required for epithelial sheet integrity, coordinated migrations during scratch-wound repair, and mechano-sensing using Magnetic Twist Cytometry. Together, these data show that α -cat phosphorylation is required for strong intercellular adhesion and tissue morphogenesis, and suggest that dynamic cycles of phosphorylation and dephosphorylation are required for the mechano-sensing properties of α -cat.

P580**Board Number: B831****Tumor Necrosis Factor induces a biphasic change in claudin-2 expression in tubular epithelial cells: potential role in barrier functions and proliferation.**Y. Amoozadeh^{1,2}, Q. Dan^{1,2}, J. Xiao^{1,2}, F. Waheed^{1,2}, K. Szaszi^{1,2};¹Keenan Research Center for Biomedical Science, St Michael's Hosp, Toronto, ON, ²Department of Surgery, University of Toronto, Toronto, ON

Tubulointerstitial inflammation is a condition associated with acute and chronic kidney disease. It is triggered by harmful stimuli including ischemia-reperfusion, albuminuria, toxins and infectious agents. The inflammatory cytokine Tumor Necrosis Factor- α (TNF- α) is now recognized as a key mediator of kidney injury. We have previously shown that TNF- α induces cytoskeleton remodeling in tubular epithelial cells, and alters their permeability. To gain insight into the mechanisms of this latter effect, we used the proximal tubule cell line LLC-PK1 to explore changes in tight junction protein expression. We show that short-term (1-3h) TNF- α treatment induced an increase in the total and cell surface levels of the channel forming claudin-2. In contrast, long-term (>8h) TNF- α treatment induced a marked downregulation of claudin-2, which was accompanied by an increase in claudin-1, 4 and 7 expression. Interestingly, the early increase and the late decrease in claudin-2 expression differed both in the mechanisms and the signaling pathways involved. The late decrease involved changes in mRNA levels and promoter activity, suggesting reduced synthesis of claudin-2. In contrast, the early increase was independent of these, suggesting a decrease in protein degradation. As Epidermal Growth Factor (EGF) has been shown to reduce claudin-2 expression in tubular cells, and our previous results indicated that TNF- α transactivates the EGF receptor, we asked whether EGF receptor signaling might play a role in the effect of TNF- α on claudin-2. We found that the early increase but not the late decrease in claudin-2 expression required the EGF receptor and its downstream effectors ERK, Rho and Rho kinase. Next we asked whether TNF- α -induced changes in paracellular permeability and cell proliferation could be linked to altered claudin-2 expression. We measured Transepithelial Resistance (TER) using Electric Cell-substrate Impedance Sensing (ECIS). TNF- α exerted a biphasic effect on TER causing an initial decrease (corresponding to increased permeability) and late increase (corresponding to reduced permeability). In line with its role as a high permeability protein, claudin-2 silencing with an siRNA elevated TER, and mimicked the effect of long-term TNF- α treatment. Importantly, long-term TNF- α treatment did not induce further TER increase when claudin-2 was silenced. Finally, claudin-2 siRNA also prevented the proliferative effect of TNF- α . Taken together, our findings suggest that altered claudin-2 expression during inflammation not only impacts on barrier functions, but also changes proliferative responses of tubular cells. Claudin-2 expression could be a key factor affecting epithelial cell fate and tubular regeneration following injury.

P581**Board Number: B832****Characterization of RhoA-GTP flares in the *Xenopus laevis* epithelium.**R.E. Stephenson¹, T.R. Arnold¹, T. Higashi¹, A.L. Miller¹;¹Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI

Precise regulation of the small GTPase RhoA is important for the proper formation and maintenance of cell-cell junctions in epithelial cells. While active RhoA (RhoA-GTP) is known to rapidly and transiently accumulate during processes like cytokinesis and wound healing, the population of active RhoA at cell-cell junctions was previously thought to be relatively stable over time. Recent work from our lab has demonstrated that knockdown of the scaffolding protein Anillin in the epithelium of gastrula-stage *Xenopus laevis* embryos results in loss of cell-cell junction integrity, which is accompanied by acute, local accumulations, or flares, of RhoA-GTP at cell-cell junctions. Despite the dramatic nature of the RhoA-GTP flares, their cause and the consequence for the cell remain unclear. Here we show that perturbation of the RhoA regulator Guanine nucleotide Exchange Factor-H1 (GEF-H1) by injection an antisense morpholino or by overexpression of a dominant negative form of GEF-H1 results in similar RhoA-GTP flares. We examined tight junction behavior during RhoA-GTP flares using live imaging of mRFP-ZO-1, a fluorescently tagged tight junction plaque protein, and found that RhoA-GTP flares are preceded by discontinuities in mRFP-ZO-1. Following the flare, mRFP-ZO-1 signal becomes continuous again, suggesting that RhoA-GTP flares serve to repair junctions. RhoA-GTP flares are accompanied by protrusions of the plasma membrane and accumulations of filamentous actin in the protruding cell and its neighbor. We are currently working to characterize the molecular events surrounding these RhoA-GTP flares, which may give us insight into how junctions are maintained and repaired during dynamic processes like epithelial cell division and morphogenesis.

P582**Board Number: B833****Diurnal variations in matrix metalloproteinase expression and tight junction integrity in *Xenopus laevis* corneal epithelium.**A.F. Wiechmann¹, B.P. Ceresa², E.W. Howard¹;¹Department of Cell Biology, Univ Oklahoma Hlth Sci Ctr, Oklahoma City, OK, ²Pharmacology and Toxicology, University of Louisville School of Medicine, Louisville, KY

Introduction: The corneal epithelium (CE) provides a permeability barrier largely due to the presence of tight junctions between neighboring surface CE cells. As the surface CE cells mature, they must actively desquamate to maintain tissue homeostasis. Matrix metalloproteinases (MMPs) are expressed in the CE, and are known to target junctional proteins in other systems. The objective of this study was to investigate the potential temporal and spatial inverse relationship of MMP expression and tight junction integrity in the *Xenopus laevis* CE.

Methods: Corneas were obtained from adult *Xenopus laevis* frogs during the light or dark period of the day/night cycle and examined by double label confocal immunohistochemistry.

Results: Expression of MMP-2, tissue inhibitor of MMP-2 (TIMP-2) and membrane type 1-MMP (MT1-MMP) and the tight junction proteins occludin and claudin-4 were present in the CE. Occludin and claudin-4 expression was generally intact on the lateral membranes during the light period, but was frequently disrupted in small clusters of cells during the dark period. MMP-2 expression was often elevated in a mosaic pattern at night and associated with clusters of desquamating surface cells. The MMP-2 binding partners, TIMP-2 and MT1-MMP were also localized to surface corneal epithelial cells during both the light and dark phases, with TIMP-2 tending to be elevated during the daytime.

Conclusions: MMP-2 protein expression is elevated in a mosaic pattern in surface corneal epithelial cells during the nighttime in *Xenopus laevis*, and may play a role in homeostatic surface cell desquamation by disrupting intercellular junctional proteins.

P583

Board Number: B834

Regulation of fibrotic signaling by desmosomal armadillo proteins in cardiac tissue.

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The process of fibrosis, described as accumulation of myofibroblasts and excessive deposition of extracellular matrix components, is a key development in the progression of multiple different types of cardiac disease. Nevertheless, little is known about the molecular mechanisms which cause the onset of fibrosis in cardiac disease. Fibrosis is a significant component of arrhythmogenic cardiomyopathy (AC), a genetic disorder characterized by replacement of healthy cardiomyocytes (CMs) with fibrous tissue, leading to arrhythmia and in certain cases, sudden death. AC is often characterized as a “disease of the desmosome”, as mutations for all obligate desmosome proteins have been found in cases of AC, including the desmosome armadillo proteins Plakophilin-2 (PKP2) and Plakoglobin (PG). PKP2 and PG are multi-functional proteins involved in both mechanical stabilization of the cardiac area composita, as well as mediation of desmosome-related signaling pathways. We have determined that loss of PKP2 or PG in neonatal CMs causes an aberrant increase in gene expression of pro-fibrotic stimuli such as transforming growth factor beta 1 (TGF-beta1) and Interleukin-6 (IL-6). In addition, p38 MAPK, a known mediator of inflammatory fibrosis, is activated upon loss of PKP2/PG. We hypothesize that mutation or loss of PKP2 or PG cause the recruitment and activation of cardiac fibroblasts via pro-fibrotic TGF-beta and p38MAPK signaling, resulting in pathological fibrosis characteristic of AC. Indeed, conditioned media from PKP2-silenced CMs causes an increase in fibronectin gene expression by freshly isolated cardiac fibroblasts. Our future experiments will investigate whether inhibition of TGF-beta or p38MAPK signaling can alleviate fibrotic gene production. By highlighting a novel link between desmosome armadillo proteins and pro-fibrotic signaling in cardiac tissue, this study provides mechanistic insights into the pathogenesis

of AC, as well as advances our knowledge of potential therapeutic targets for combating fibrosis in multiple different types of heart disease or injury.

P584

Board Number: B835

Regulation of epithelial morphogenesis by a tight junction (TJ)-apical complex.

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Epithelial cells adhere to each other by tight junctions (TJs) to form cell sheets, which is a critical step in epithelial morphogenesis. We recently discovered that a network of microtubules exists just below the apical membrane of the epithelial cell sheet. Because this apical microtubule network appears to be connected to TJs and associated with actin and keratin filaments, we defined the TJ and its associated membrane and apical structures as the "TJ-apical complex." The TJ-apical microtubules represent one of the layered cytoskeletal networks within the TJ-apical complex. Gel overlay assays of isolated TJ fractions on microtubules in the presence of taxol revealed four TJ-associated microtubule-binding proteins (TJ-MAPs): TJ-MAP1, TJ-MAP2 (cingulin), TJ-MAP3, and TJ-MAP4. A knockdown (KD) analysis for cingulin revealed that cingulin-KD partially disrupted the association between microtubules and TJs. More recently, we found a similar effect of knocking out TJ-MAP3. We further found that TJ-MAP3 critically influences the TJ-actin organization by regulating the phosphorylation of myosin, suggesting a functional link between the microtubules and the actomyosin ring at TJs. In addition, we found that TJ-MAP3 contributes to the apical constriction mediated by Rho/ROCK signaling and functionally regulated by microtubules.

Based on these findings, we propose the novel idea that the planar network of apical microtubules is linked to circumferential actin rings at the TJ-apical complexes. Future studies will explore the role of the TJ-apical complex in the general context of all cytoskeletons, including those based on microtubules, actin, and keratin filaments. We propose that the TJ-apical complex is critical for organizing the TJ and apical membrane systems, including the location and function of membrane proteins that play essential roles in epithelial cell sheet functions, and thus in the regulation of biological systems.

P585**Board Number: B836****Bidirectional crosstalk between retinal pigment epithelium (RPE) and endothelial cells regulates RPE tight junctions.**I. Benedicto¹, G. Lehmann-Mantaras¹, M. Ginsberg², D.J. Nolan², S. Rafii³, E. Rodriguez-Boulan⁴;¹Department of Ophthalmology, Weill Cornell Medical College, New York, NY, ²Angiocrine Biosciences, New York, NY, ³Department of Genetic Medicine, Weill Cornell Medical College, New York, NY,⁴Department of Ophthalmology, Dyson Vision Research Institute, Weill Medical College of Cornell University, New York, NY

In the eye, the retinal pigment epithelium (RPE) separates the neural retina from the fenestrated choroid endothelium. RPE tight junctions (TJs) are key for the correct maintenance of the outer blood-retinal barrier. Whereas it has been shown that RPE TJs are affected by factors secreted by the neural retina, it is not known whether RPE TJs are also regulated by choroid endothelial cells (ECs). Recent studies have demonstrated that ECs play key instructive roles in the differentiation and maintenance of parenchymal cells in several body organs. Thus, we studied whether ECs regulate RPE TJ function.

We performed co-culture experiments on Transwell inserts using human fetal RPE (hFRPE) and either human umbilical vein ECs (HUVECs) or mouse primary choroid ECs, both expressing the adenoviral protein E4. E4-expressing ECs are able to survive in the absence of endothelial factors or serum. hFRPE were seeded on top of the filter and ECs were seeded on the bottom chamber of the insert. The effect of ECs on hFRPE transepithelial electrical resistance (TER) was assessed at different time points during both de novo TJ formation and TJ recovery after disruption by TNF α treatment. As controls, several cell lines were used instead of ECs. Alternatively, co-cultures were carried out with ECs that had been previously exposed to polarized hFRPE conditioned medium. Expression levels of the TJ-associated proteins claudin-1, -2, -3, -9, -10b, -12, -15, -16, -19 and occludin were analyzed in hFRPE at different time points by real time PCR. The effect of the epidermal growth factor receptor (EGFR) inhibitor AG1478 on hFRPE TER and claudin levels was also evaluated.

hFRPE co-cultured with ECs presented a significantly increased TER both in the presence or absence of serum. This effect was not detected when other cell types were used instead of ECs. The increase in TER was faster when ECs had been previously primed with hFRPE. We also observed that the presence of ECs induced a specific decrease in hFRPE claudin-2 mRNA levels. EGFR inhibition significantly reduced EC-mediated changes in hFRPE TER and claudin-2 levels.

Our results suggest that choroid endothelium modulates RPE TJ function. In addition, the fact that the effect on TER was faster when ECs were previously primed with hFRPE suggests a bidirectional communication model in which ECs receive a signal from RPE which in turn enhances their ability to modulate RPE TJs. Our inhibition assays suggest that EGFR could participate in such crosstalk. This model fits well with the observed synchronized development of RPE and choroid endothelium.

P586**Board Number: B837****The scaffolding protein Anillin contributes to epithelial cell-cell junction protein dynamics.**T.R. Arnold¹, R.E. Stephenson¹, T. Higashi¹, A.L. Miller¹;¹Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI

Intact cell-cell junctions and proper cell division are critical for both tissue development and homeostasis, while failures in these processes contribute to disease. Anillin is a well-characterized scaffolding protein that recruits and stabilizes components of the contractile ring during cell division. We recently discovered that a population of Anillin localizes to epithelial cell-cell junctions and is important for their proper organization and function, though the mechanism is still unclear. We investigated the dynamics of junction proteins when Anillin was perturbed in the intact epithelium of *Xenopus laevis* gastrula-stage embryos. Using fluorescence recovery after photobleaching (FRAP), we found that the tight junction protein ZO-1 recovers faster when Anillin is knocked down and slower when Anillin is overexpressed, suggesting that Anillin stabilizes ZO-1 protein dynamics and may contribute to junction structure and function by regulating the dynamics junctional proteins. Further, we observed that knockdown of Anillin leads to local, acute discontinuities in ZO-1, followed by localized flares of active RhoA, and then re-establishment of ZO-1. This suggests that the increased frequency of active RhoA flares we previously reported in Anillin knock down embryos is likely a response to loss of junction integrity and may indicate that transient localized activation of RhoA serves to repair or remodel junctions. Additionally, Anillin is enriched at the cytokinetic contractile ring, a site of high junctional tension. Because changes in tension and Anillin levels can alter junction protein dynamics, we hypothesize that junction proteins near the furrow may exhibit altered FRAP. We are currently investigating whether cell-cell junction proteins in dividing cells have altered dynamics at the cleavage furrow relative to polar regions. Our studies provide evidence that Anillin contributes to normal junction protein dynamics and that local activation of RhoA precedes re-establishment of junctions when Anillin is knocked down.

P587**Board Number: B838****The organophosphate pesticide methamidophos disrupts de blood testis barrier by inducing the phosphorylation of the tight junction proteins occludin and ZO-2.**J. Ortega-Olvera¹, B. Chávez², B. Quintanilla-Vega³, L. Gonzalez-Mariscal⁴;¹Physiology, Biophysics and Neuroscience, CINVESTAV, Mexico City, Mexico, ²Infectomics and Molecular Pathogenesis, CINVESTAV, Mexico City, Mexico, ³Toxicology, CINVESTAV, Mexico City, Mexico,⁴CINVESTAV, Mexico City, Mexico

Methamidophos (MET) is an organophosphate pesticide widely used in agriculture in developing countries. MET has been associated with male reproductive alterations including a decrease in sperm quality and fertilization ability, as well as sperm DNA damage. The blood-testis barrier (BTB) plays a crucial role in spermatogenesis, since it seals the paracellular route between Sertoli cells and protects post meiotic germ cells from the immune system present in the systemic circulation. The BTB is constituted by several types of junctions including tight junctions (TJ) that may be targets of MET. Here we have explored if MET disrupts the BTB by targeting TJ. To explore this, CD1 mice were treated for 4 consecutive days with MET (5 mg/kg body weight) and sacrificed one day later. We observed that MET treatment induced the appearance of a leaky BTB by using a biotin tracer and that the paracellular space between Sertoli cells is widened as observed by transmission electron microscopy. These results prompted us to analyze the effect of MET on TJ proteins that form the BTB: claudin 11, occludin and ZO-2. In frozen testis sections from control and MET treated animals, claudin-11, occludin and ZO-2 circumscribed each Sertoli cell near the basement membrane. However, when seminiferous tubules were isolated we observed that the ZO-2 chicken fence pattern was disturbed by numerous breaks in the network. Since MET induces the addition of phosphate-adducts to proteins, we then explored if the treatment with MET increased the amount of phosphorylated proteins in the testis. We observed that this is the case, and further demonstrated by using a phosphate affinity SDS-PAGE that ZO-2 and occludin become hyperphosphorylated after MET treatment. In conclusion, our results indicate that MET induces the rupture of the BTB probable by increasing the phosphorylation of the TJ proteins ZO-2 and occludin.

P588

Board Number: B839

Using microfluidic approaches to study gap junction intercellular communication.

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Intercellular communication is vital for multicellular life. In addition to endocrine and paracrine signaling, gap junctions mediate direct metabolic and electrical coupling between cells. The disruption of these junctions brings drastic consequences. Without gap junction intercellular communication (GJIC), the contraction of the heart becomes uncoordinated, Schwann Cells fail to maintain peripheral nerves and macrophages no longer impart signals upon tissues, for example. With such diversity in roles, it follows that GJIC is tightly regulated both by gating and by downregulation from the plasma membrane.

To date, a variety of techniques have been used to assay GJIC. While valuable, some established assays (e.g., microinjection, patch clamping or gap-fluorescence recovery after photobleaching) are technically difficult or only measure single-cell effects – a laborious route for obtaining rigorous, well-powered conclusions. While the preloading assay offers an opportunity to easily study many cells, this technique requires proper gap junction formation and there is evidence for nonspecific calcein transfer between

cells. Lastly, the population-level scrape loading assay is confounded by the fact that the cells studied must be torn apart for loading.

When met with these difficulties at the 2014 MBL physiology course, we established a microfluidics assay to measure GJIC and the effects of two different drugs: carbenoxolone (purported to gate gap junctions) and phorbol 12-myristate 13-acetate (PMA) (a PKC activator thought to remove gap junctions from the plasma membrane). Using a t-shaped microchannel, we specifically loaded a fraction of cells with Calcein AM and measured its diffusion through gap junctions (obtaining a diffusion coefficient similar to that measured by other implementations of this technique). As expected, carbenoxolone-mediated gap junction gating led to a drastic inhibition of GJIC, while PMA-mediated gap junction downregulation led to an intermediate effect. Also while at the course, we met a TA who recently published how to adapt a commercially available CellASIC system to study GJIC by hydrodynamic focusing. Considering the reproducibility of our findings using microfluidic approaches and the ease of assessing the extent of GJIC over many cells at once, we argue that microfluidics serves as an ideal method to measure GJIC in living cells.

P589

Board Number: B840

Development of an angulin binder and its tricellular tight junction-modulating activity.

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Epithelial cell sheets and tissues possess a tight junction (TJ) which almost perfectly seal the intercellular space against uptake of unwanted large solutes, but also of large drug molecules. The TJ between two adjacent cells is named bicellular TJ (bTJ) while that at the meeting point of three cells is the tricellular TJ (tTJ). One strategy for oral drug delivery is to modulate the TJ seal and by this to enhance the permeation of drugs. Several classes of TJ modulators have been identified so far including chelators, bile acids, fatty acids, and an enterotoxin fragment. All of them modulate the bTJ, but permeation enhancers targeting the tTJ have not been fully developed so far. The tTJ comprises a tetra-transmembrane protein, tricellulin, and lipolysis-stimulated lipoprotein receptor (also known as angulin-1), a transmembrane protein of the angulin family which consists of three members: angulin-1, -2, and -3. Angulin-1 is a receptor for *Clostridium perfringens* iota toxin (Ib). This suggests that a receptor-binding component of Ib might modulate the epithelial barrier by interaction with the tTJ. Here, we investigated whether angulin may be a target for modulation of the tTJ seal using the putative receptor-binding domain corresponding to amino acids 421–664 of Ib (Ib421–664). Ib421–664 bound to angulin-1-expressing cells without causing cytotoxicity and colocalized with angulin-1. Ib421–664 interacted with angulin-1 and -3, but not with angulin-2 and decreased epithelial integrity in angulin-1- or -3-expressing, but not in angulin-2-expressing cells. Thus, Ib421-664 appears to act as an angulin-1 and -3 binder. Two-

path impedance spectroscopy showed that Ib421-664 decreased the electrical resistance of the TJ pathway in the intestinal monolayer-forming cell line HT-29/B6. Ib421-664 enhanced the permeation of solutes across these cells and immuno-fluorescence data indicated that the angulin binder may allow solutes to pass through the tTJ. Solute absorption was enhanced by Ib421-664 also in rat jejunum. In conclusion, Ib421-664 is the first compound shown to bind to tTJs and to act in vitro and in vivo as an tTJ modulator providing a new mechanism for enhancing drug delivery.

Cadherins and Cell–Cell Interactions

P590

Board Number: B841

Quantifying the time-dependent strengthening of cell-cell adhesion using a monolayer cell-binding assay.

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Cell adhesion affects the development and maintenance of tissue integrity. Quantification of cell adhesion is important in biomedical research because of the importance of cell adhesion in a number of cellular processes, including tumor metastasis. We have designed a novel monolayer cell-binding assay yielding data comparable to, but more rapid and less expensive than, the dual pipette assay previously used in this work. Unlabeled cells are centrifuged in 25mm wells at 5000 rpm to form a confluent monolayer. Fluorescently-labelled probe cells are then brought into contact with the monolayer for defined periods of time, at the end of which the monolayers are subjected to a gyratory shear. Adhesion is quantified in terms of the number of labeled fluorescent probe cells still bound after the gyratory shear. Here, we compare two methods to quantify bound probe cells: 1) a densitometric method using iVision software to count fluorescent pixels in multiple fields of view from monolayers photographed using fluorescence microscopy, and 2) a fluorimetric approach using a Synergy HT fluorimeter and KC4 software to quantify fluorescence solubilized directly from the test wells. Both methods showed strong correlations between the measured fluorescent signal and the actual number of fluorescent cells present ($R^2 = 0.99629$ and 0.9145 , respectively), and are thus comparably accurate. However, the fluorimetric method is faster and allows us to collect more data in less time.

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P591**Board Number: B842****Cellular distribution and expression of cell adhesion molecules (CAMs) in pancreatic islet cells of obese and diabetic mice.**V.T. Falcão¹, R.B. Oliveira², D.A. Maschio², C.P. Carvalho³, C.B. Collares-Buzato²;¹College of Nursing, University of Pernambuco, Recife - PE, Brazil, ²Department of Biochemistry and Tissue Biology, University of Campinas (UNICAMP), Campinas - SP, Brazil, ³Department of Biosciences, Federal University of São Paulo (UNIFESP), Santos - SP, Brazil

Intercellular junctions (IJs) and their CAMs participate in important cellular processes such as cell adhesion, growth/death and signaling. In the endocrine pancreas, IJs play a role in regulating islet cytoarchitecture, insulin biosynthesis and secretion. In this work, we investigated the islet histology and cellular distribution and content of CAMs (i.e., E-, N-, VE-cadherins, ZO-1, α - and β -catenins) in the endocrine pancreas of C57BL/6/JUnib mice fed a high-fat (HF) diet (21% lipids, w/w) for a prolonged time period (8 months). After HF diet exposure, mice became obese and displayed marked metabolic disturbances indicative of establishment of type 2 diabetes mellitus (T2DM), such as marked peripheral insulin resistance and hyperglycemia (in fasted and fed states), and moderate postprandial hyperinsulinemia in comparison with the control mice (fed on regular diet containing 4.5% lipids, w/w). Isolated pancreatic islets of HF-fed mice showed a significant impairment of glucose-stimulated insulin secretion in comparison with control islets. Histology of the endocrine pancreas revealed no marked changes in islet morphology and cytoarchitecture between animal groups, although HF-fed mice showed a 57% increase in the relative islet volume (per total pancreas) in comparison with controls, indicative of beta-cell mass expansion. As shown by immunohistochemistry and Western Blot, E-, N-cadherins, ZO-1, and catenins, were expressed at the intercellular contact site of endocrine cells while VE-cadherin was restricted to the islet vascular compartment. A cellular redistribution of N- and E-cadherins and α -catenin (from the contact region to the cytoplasm) in pancreatic endocrine cells and a significant increase in VE-cadherin islet content were seen in diabetic mice as compared to controls. No significant differences in islet immunoreaction for the other CAMs were observed between the experimental groups. In conclusion, CAMs are expressed by endocrine and endothelial cells of pancreatic islets and, in particular, the distribution and content of N-, E- and VE-cadherins as well as α -catenin are significantly altered in islet cells of obese and diabetic mice, that may have a relevance in the T2DM pathogenesis. This research was approved by the Ethics Committee of Experimental Animal IB/UNICAMP (# 3122-1) and financially supported by FAPESP/Brazil (# 2010/50789-1).

P592**Board Number: B843****Regulation of Cadherin-Mediated Cell Adhesion and Lumen Formation by the Tyrosine Phosphatase, PTPN14.**P. Dimarco¹, A. Gladden¹;¹Univ Texas M.D. Anderson Cancer Ctr, Houston, TX

Cell-cell adhesion is mediated through dynamic regulation of cell junctions including adherens junctions (AJ). The AJ comprises the E-cadherin/ β -catenin protein complex that is assembled in the Golgi and transported to cell-cell contacts. Multiple regulatory mechanisms, including tyrosine phosphorylation, influence how the E-cadherin/ β -catenin complex promotes adhesion. The non-receptor tyrosine phosphatase, PTPN14, is a member of the Band 4.1, Ezrin, Radixin, Moesin (FERM) family of proteins that plays a critical role in linking membrane proteins to the cell cytoskeleton, thereby aiding in the communication of extracellular signals to the cytoskeleton. PTPN14 is mutated or deleted in several human cancers, including colorectal cancer, suggesting that PTPN14 activity may be involved in tumor development and/or progression. Previous work identified β -catenin as a PTPN14 substrate, but how PTPN14 regulates β -catenin-mediated adhesion or signaling is not known. The observations that PTPN14 is altered in cancer, coupled with its potential role in AJ regulation, led us to examine the function of PTPN14 in the non-malignant intestinal cell line, Caco-2BBE. We found that endogenous PTPN14 co-localizes with E-cadherin during initial cell junction formation and with both E-cadherin and the tight junction (TJ) protein ZO-1 in both mature cell contacts and in 3D cysts. PTPN14 knockdown (kdPTPN14) disrupted ZO-1 localization, actin cytoskeleton organization and formation of functional AJ and TJ. Furthermore, kdPTPN14 cells were unable to form lumens in 3D cultures. To determine how PTPN14 regulates cell contacts and lumen formation, we examined the levels of phosphorylation at β -catenin tyrosine residues known to be phosphorylated by upstream kinases. Notably, kdPTPN14 cells displayed increased levels of β -catenin Y654 phosphorylation, but no change in Y142 or Y489 phosphorylation. Src kinase phosphorylates β -catenin Y654, triggering disruption of the β -catenin/E-cadherin complex. Live-cell imaging revealed that whereas control cells displayed primarily intramembranous movement of E-cadherin at cell:cell contacts, PTPN14 depleted cells showed a significant increase in E-cadherin movement on and off of the membrane at cell:cell contacts. FRAP analysis confirmed increased E-cadherin movement and recovery after photobleaching, indicating that PTPN14 stabilizes E-cadherin complexes at cell:cell contacts. E-cadherin movement can be restored by expression of full-length PTPN14 or treatment with a Src inhibitor, thus providing evidence that PTPN14 activity opposes Src-mediated disruption of the AJ. These results suggest that PTPN14 promotes cell adhesion, tissue organization and could be utilized therapeutically to prevent tumor progression.

P593**Board Number: B844****Twist1-induced epithelial dissemination requires E-cadherin and is regulated by heterotypic cell-cell dynamics.**E. Shamir¹, E. Pappalardo², P. Tran³, J. Bader², A. Ewald¹;¹Department of Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD, ²Department of Biomedical Engineering, Johns Hopkins University, Baltimore, MD, ³Radiation Oncology and Molecular Radiation Sciences, Johns Hopkins University School of Medicine, Baltimore, MD

Dissemination of epithelial cancer cells represents the first step in metastatic spread. One postulated mechanism for dissemination is activation of an epithelial to mesenchymal transition (EMT), in which repression of the cell adhesion gene E-cadherin (E-cad) is considered the driving molecular event. We sought to test the sufficiency of single gene perturbations to induce dissemination out of primary mouse mammary epithelium. Deletion of E-cad disrupted simple architecture and morphogenesis but, contrary to expectation, rarely resulted in dissemination. In contrast, expression of the EMT transcription factor Twist1 induced rapid dissemination of cytokeratin+ epithelial cells. A core concept in EMT is that cells lose epithelial characteristics, such as tight cell-cell adhesion, and acquire mesenchymal characteristics to invade as single cells. However, we found that Twist1 did not significantly regulate epithelial-specific genes, such as E-cad. Rather, Twist1 induced dramatic transcriptional changes in extracellular compartment and cell-matrix adhesion genes. Surprisingly, we observed Twist1+ disseminating cells with membrane-localized E-cad, and complete knockdown of E-cad strongly inhibited Twist1-induced single cell dissemination. Dissemination can therefore occur through an innately epithelial migratory program.

In clinical management of ductal carcinoma in situ, focal disruptions in the myoepithelium, the basal mammary epithelial layer, serve as a negative prognostic indicator. Interestingly, we found that constitutive Twist1 expression induced dissemination of both inner luminal and outer myoepithelial cells and abnormal ingression of myoepithelial cells, resulting in gaps in myoepithelial coverage at the basal surface. We next developed mouse models to restrict Twist1 to distinct mammary lineages. Twist1 expression in the myoepithelial compartment induced cell autonomous myoepithelial dissemination. In contrast, Twist1 expression in the luminal compartment resulted in almost no dissemination. Using fluorescent reporters to distinguish Twist1+ and Twist1- cells, we observed that normal myoepithelial cells appear to contain Twist1+ luminal cells protruding into the extracellular matrix. Myoepithelial cells display a similar response to invasive E-cad- luminal cell behavior. Taken together, our data supports the hypothesis that normal myoepithelial cells dynamically block luminal cell escape. We now seek to test the molecular basis of myoepithelial barrier function and have validated shRNAs to knock down myoepithelial-specific genes important for contractility, cell-cell adhesion, and the intermediate filament network. We expect that perturbation of myoepithelial function will enable E-cad- and Twist1+ luminal cell dissemination.

P594**Board Number: B845****Nanoscale organization of cadherin-mediated adhesion complexes formed on planar cadherin-coated surfaces.**C. Bertocchi¹, T. Sailov¹, M. Baird², R. Mege³, B. Ladoux^{3,4}, M.W. Davidson⁵, P. Kanchanawong^{1,6};¹MBI, National University of Singapore, Singapore, Singapore, ²Florida State University, Tallahassee, FL,³Institut Jacques Monod, Université Paris Diderot CNRS, Paris, France, ⁴Mechanobiology Institute,National University of Singapore, Singapore, Singapore, ⁵National High Magnetic Field Laboratory andDepartment of Biological Science, Florida State University, Tallahassee, FL, ⁶Department of Biomedical

Engineering, National University of Singapore, Singapore, Singapore

Cadherin-mediated junctions are of fundamental importance for multicellularity and tissue homeostasis. Although cadherins are part of a supramolecular complex that performs diverse functions such as mechanotransduction, signaling and dynamic regulation and linkages to the actin cytoskeleton, still much remains unknown about the ultrastructural basis of these functions. Although superresolution microscopy offers a potential avenue for dissecting the nanoscale organization within these complexes, the highly variable 3-D geometry of the native cell-cell junctions is sub-optimal for high resolution optics. Therefore, we employed a planarized substrate which presents uniformly oriented cadherin extracellular domains that mimic many attributes of true cell-cell junctions such as cadherins clustering, recruitment of canonical cadherin associated proteins, and importantly the connection to the actin cytoskeleton. To elucidate the nanoscale architecture underlying distinct cadherin-mediated complexes, we applied superresolution microscopy techniques to determine proteins position and orientation in C2C12 myoblast (N-cadherin) and MDCK cells (E-cadherin) cultured on respective cadherin biomimetic surfaces. We observed that distinct proteins within the cadherin-mediated complexes appear to be differentially partitioned along the vertical (z) axis at the nanoscale. Proteins that reside in close proximity to the cadherin cytoplasmic tails (N-cadherin: $z = 47.4 \pm 5.9\text{nm}$; E-cadherin: $z = 46.9 \pm 4.7\text{nm}$, relative to the substrate) include p120-catenin ($z = 47.9 \pm 8.3\text{nm}$ in C2C12; $45.0 \pm 4.3\text{nm}$ in MDCK) and α -catenin (n-terminal probe) ($z = 47.0 \pm 10.3\text{nm}$ in C2C12; $41.5 \pm 7.5\text{nm}$ in MDCK). The actin cytoskeleton is distinctly separated from the cadherin-associated partition by $\sim 30\text{nm}$, and is closely associated with actin-binding proteins such as α -actinin, eplin, VASP, and zyxin. Interestingly, vinculin was observed to span between these two vertical partitions, suggesting that it is oriented in a polarized manner, and likely in an open and active conformation. Similar hierarchies of nanoscale protein organization between the two different cell types suggest a commonality between the nature of the physical links between actin and E-/N-cadherin. However, we observed a greater extent of vertical stratification in N-cadherin-based complexes. In particular, vinculin appears to be highly extended, with at least $\sim 30\text{nm}$ contour length, consistent with the large and elongated morphology of the cadherin clusters in the more contractile myoblast cells. Our results suggest how cadherins and associated proteins are assembled at the nanoscale level on a planar format, which likely recapitulates how they are organized in native cell-cell contacts to interface with the actin cytoskeleton.

P595**Board Number: B846****Actin dynamics modulate mechanosensitive immobilization of E-cadherin at adherens junctions.**V. Viasnoff^{1,2}, W. Engl¹, J. Thiery^{3,4}, X. Gao¹;¹MBI/DBS, Mechanobiology Institute, Singapore, Singapore, ²UMI3639, CNRS, Singapore, Singapore,³Biochemistry, NUS, Singapore, Singapore, ⁴IMCB, Astar, Singapore, Singapore

Mechanical stress is increasingly being shown to be a potent modulator of cell–cell junctional morphologies in developmental and homeostatic processes. Intercellular force sensing is thus expected to be an important regulator of cell signalling and tissue integrity. In particular, the interplay between myosin contractility, actin dynamics and E-cadherin recruitment largely remains to be uncovered. We devised a suspended cell doublet assay to quantitatively assess the correlation between myosin II activity and local E-cadherin recruitment. The single junction of the doublet exhibited a stereotypical morphology, with E-cadherin accumulating into clusters of varied concentrations at the rim of the circular contact. This local recruitment into clusters derived from the sequestration of E-cadherin through a myosin-II-driven modulation of actin turnover. We also show the similarities between E-cadherin recruitment induced by internal contractions or external mechanical stimuli. We exemplify how the regulation of actin dynamics provides a mechanism for the mechanosensitive response of cell contacts.

Ref: Engl *et al*, Nature Cell Biology 16(6) 2014**P596****Board Number: B847****FAK-induced expression of N-cadherin regulates mechanosensitive cell cycling.**K.L. Mui¹, Y. Bae², L. Gao^{1,3}, S. Liu^{1,2}, T. Xu², G.L. Radice⁴, C.S. Chen^{5,6}, R.K. Assoian¹;¹Cell and Molecular Biology Graduate Group, University of Pennsylvania, Philadelphia, PA,²Pharmacology, University of Pennsylvania, Philadelphia, PA, ³Bioengineering, University ofPennsylvania, Philadelphia, PA, ⁴Thomas Jefferson Univ, Philadelphia, PA, ⁵Biomedical Engineering,Boston University, Boston, MA, ⁶Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, MA

Cell-cell and cell-matrix adhesions transduce mechanical signals from the extracellular environment to regulate fundamental cellular processes. While it is well established that cell-matrix adhesions stimulate cell cycling, the effect of cadherin-based cell-cell adhesion on proliferation is not as well understood. We now demonstrate that matrix stiffness controls the crosstalk between cell-matrix and cell-cell adhesion complexes by promoting expression of N-cadherin through a FAK-Cas-Rac signaling pathway. In vivo mouse studies reveal a strong upregulation of N-cadherin in injured arteries that have undergone tissue

stiffening. Complementary in vitro studies examining vascular smooth muscle cells (VSMCs) cultured on polyacrylamide hydrogels matched to the elastic moduli of healthy and injured arteries demonstrate that N-cadherin protein expression increases with stiffness, and that this effect requires stiffness-regulated activation of FAK which in turn selectively activates Cas and ultimately Rac. To determine the functional importance of stiffness-induced expression of N-cadherin, we examined the consequence of N-cadherin engagement on cell proliferation. Using micropatterned islands to control cell shape and cell-cell contact, we show that N-cadherin-mediated contact overrides the spreading requirement for VSMC cycling in vitro. Conditional knockout of N-cadherin in mouse VSMCs also demonstrates that N-cadherin is essential for proliferation after arterial injury. Furthermore, in vivo deletion of FAK in VSMCs phenocopies the effect of N-cadherin deletion on cell proliferation in response to injury. Therefore, we conclude that N-cadherin is an essential downstream effector of FAK during stiffness-induced cell cycling in vivo. Overall, our studies demonstrate that matrix stiffness is an important regulator of N-cadherin expression and that, in turn, N-cadherin-dependent signaling is essential for stiffness and FAK-dependent proliferation.

P597

Board Number: B848

A positive feed-back loop between N-cadherin and FGFR regulates cell-cell cohesion and cell migration.

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In addition to its known function in adhesion, N-cadherin initiates signal transduction by interacting with several signaling proteins. A functional interaction between Ncad and Fibroblast Growth Factor Receptor (FGFR) during tumor cell migration and neurite outgrowth has been reported. How this interaction occurs, how the signal is transduced and what are the consequences for the cell remain open questions. We show that FGFR1 expression reduces the migration of Ncad expressing cells on Ncad-Fc and promotes their collective migration on fibronectin. Both proteins are co-recruited at cell-cell contacts supporting a crosstalk between Ncad and FGFR signaling in regulating cell adhesion and migration. Biosensor and co-immuno-precipitation analyses revealed an interaction between Ncad and FGFR ectodomains. Analysis by FRAP of the mobility of FGFR and Ncad at cell-cell contacts shows that the dynamics of FGFR closely follows that of Ncad when they are co-expressed. The appearance of an immobile fraction of FGFR at cell-cell contacts is specific of Ncad and was not observed in FGFR/E-cad expressing cells. In turn the expression of FGFR increases the immobile fraction of Ncad and cell-cell contacts strength. These effects were blocked by inhibitors of the catalytic activity of the receptor, suggesting that Ncad recruits FGFR at cell-cell contact, the activity of which strengthens Ncad adhesions by stabilizing junctional cadherins. Cell surface biotinylation followed by cell fractionation and western blot analysis shows that FGFR stabilizes Ncad at the cell membrane by decreasing its internalization.

FGFR expression also increases the recruitment and the level of phosphorylated p120. As p120 is a well-known regulator of cadherin trafficking as well as a substrate of Src, a kinase involved in FGFR signaling, we suggest that the increased phosphorylation of p120 is a consequence of the mobilization of Src by FGFR. Accordingly, the inhibition of Src decreases the phosphorylation of p120 and its recruitment at the plasma membrane. This leads to an increase of the mobile fraction of junctional Ncad as well as of cell migration on Ncad-Fc. Based on these findings we propose a positive feed-back loop between Ncad and FGFR1 at adhesion sites controlling cell migration. FGFR recruitment by Ncad adhesions is activated then activates Src. Src phosphorylates p120 which stabilizes Ncad at the cell-cell contacts by blocking its endocytosis. This stabilization strengthens cell-cell interactions, limiting cell migration on Ncad substrates and promoting collective cell migration on fibronectin. Ongoing experiments aim at understanding the link between this regulatory loop and the mechanocoupling of cadherins to the actomyosin network instrumental in cell migration.

P598

Board Number: B849

How P-cadherin induces collective cell migration? Analysis of mechanical forces and their regulation by Rho GTPases.

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Collective cell movement is relevant for many processes in morphogenesis, tissue repair, and cancer invasion and metastasis. Both mechanical and chemical cues have to be integrated at the cellular level to allow collective behaviors. Physical interactions in multicellular populations are guided by adhesion between neighboring cells and between cells and the extracellular matrix. Cell-cell adhesion molecules, and in particular cadherins, are important for collective cell migration *in vitro* and *in vivo* and are critical for coordinating cell movement and force transmission. However, the precise mechanistic pathways through which cadherins contribute to CCM remain to be examined. We analyzed the impact of P-cadherin expression, a protein associated with tumor invasion and metastasis formation, on the migratory behavior of normal mesenchymal cells. We expressed P-cadherin in mouse C2C12 myoblasts and analyzed their migration using a “wound-healing like assay” in which migration is analyzed after removal of a physical barrier. We showed that P-cadherin expression increased the speed, polarity, persistence and directionality of migration toward the free space of both cells at the multicellular leading row and cells into the multicellular layer. We concluded that P-cadherin expression induces collective cell migration. Using traction force and monolayer stress microscopy, we demonstrated that P-cadherin expression i) increases the traction forces anisotropy at the leading edge and ii) increases inter-cellular stresses and force transmission across the cell sheet. To better understand how these mechanical signals induce collective cell migration, we studied the spatio-temporal activity of Rho GTPase. We showed that P-cadherin expression activates Rac1 and Cdc42 at cell-cell contact sites and at the leading edge of the migrating multicellular layer. The knockdown of Rac1 and Cdc42 impacted both on intercellular stresses and traction forces generation demonstrating that P-cadherin-dependent

induction of collective cell migration requires Rac1 and Cdc42 activation. In conclusion, our analysis, combining a detailed measurement of the parameters of cell migration with physical measure of the intercellular stresses and traction forces, allowed to provide evidences for a mechanical role of P-cadherin during collective cell migration.

P599

Board Number: B850

Forced-dimerization and membrane targeting of α -catenin impacts epithelial sheet migration.

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Intercellular adhesion is mediated by transmembrane cadherin receptors and their associated catenins, which together integrate the recognition of an identical molecule on a neighboring cell with their coupling to the actomyosin cytoskeleton. While forced oligomerization of a cadherin lacking the catenin-binding region is known to be sufficient to enhance adhesion to recombinant cadherin ectodomains in a flow-based assay (Yap et al., 1997), the consequences of forced lateral dimerization or higher order clustering of the full cadherin/catenin complex for F-actin recruitment, junction morphology, adhesion and collective cell migrations is not known. Towards this end, we have used the iDimerize system to force α -catenin (α -cat) interactions in the context of full-length (FL) α -cat, which exists in both cadherin/ β -catenin-associated and -free forms, as well as a truncated α -cat that cannot bind β -catenin using the α -cat negative R2/7 variant of the DLD1 colon carcinoma cell line. We find that forced lateral dimerization of FL- α -cat within the cadherin complex enhances actin recruitment to the plasma membrane and increases rate of epithelial sheet migration post-wounding, while decreasing the persistence and connectivity of individual cells within the wound front. Remarkably, targeting FL α -cat to the membrane with a myristoylation sequence fails to enhance the rate of sheet migration and cell persistence despite evidence that its forced oligomerization enhances detergent insolubility and intermediate connectivity between adjacent cells. These data suggest that mechanisms that control lateral dimerization and higher order clustering of the cadherin/catenin complex may differentially contribute to epithelial sheet migrations. (Supported by NSF to MNN and NIGMS to CJG).

P600**Board Number: B851****Loss of the cardiac cell adhesion protein alpha-T-catenin increases sensitivity to toluene-diisocyanate-induced asthma.**S. Folmsbee¹, C. Gottardi^{2,3};¹Medicine - Pulmonary, Northwestern University, Chicago, IL, ²Department of Medicine, Northwestern University, Chicago, IL, ³Department of Cell and Molecular Biology, Northwestern University, Chicago, IL

α -T-catenin (α -T-cat) is an essential mediator of cell-cell adhesion through linking the β -catenin/cadherin complex to the cytoskeleton, and α -T-cat is almost exclusively described in cardiac muscle cells. It plays an important role in maintaining cardiomyocyte hybrid junctions, which are composed of desmosomes, cadherin junctions, and gap junctions. Surprisingly, a genome-wide association study (GWAS) identified single nucleotide polymorphisms (SNPs) in α -T-cat that correlated with the incidence and severity of toluene diisocyanate (TDI) occupational asthma, which is a subtype caused by exposure to environmental irritants in the workplace. Furthermore, another GWAS has identified SNPs in α -T-cat that correlated with changes to lung function in a healthy pediatric cohort. Despite these strong clinical data, a mechanism by which a dysfunction in the cell junctions of cardiomyocytes could contribute to changes to lung function and TDI-asthma has been unclear. We have found, however, that α -T-cat is indeed expressed in lung within the cardiomyocyte sheath of pulmonary veins (PV). How PV and cardiac dysfunction might underlie asthma is not known, but α -T-cat knockout (KO) mice manifest enlarged hearts with disrupted junctions and decreased contractility. To examine changes in lung physiology due to α -T-cat, we mechanically ventilated α -T-cat KO mice. There was no difference in resistance, compliance, or elasticity in the KO mice when compared to wild-type (WT), but KO mice had a significantly increased pressure-volume curve area, which suggests loss of α -T-cat may confer a mild obstructive phenotype. To test α -T-cat's role in TDI-asthma, we used a murine model of TDI-asthma with intranasal sensitization and nebulized challenge. From this, TDI-exposed α -T-cat KO mice show increased airway hyperresponsiveness to methacholine by plethysmography when compared to WT mice. Interestingly, bronchoalveolar lavage revealed only a mild macrophage-dominant inflammation that was not significantly different between WT and KO mice. Further analysis of histological sections by H&E revealed no difference between WT and KO mice in airway inflammation. Based on these data, we suspect α -T-cat dysfunction may contribute to asthma through a mechanism independent of inflammation, specifically decreased cardiac cell electrical coordination that leads to decreased contractility and increased airway edema. This process may occur because α -T-cat connects hybrid junctions through its binding to the desmosomal protein plakophilin-2 (PKP2), but the cellular function of this interaction is unknown. In future studies, we will examine the role of PKP2 binding in junction formation and gap junction coupling in cardiac cells in vitro.

P601**Board Number: B852****Force-dependent binding of vinculin to α -catenin constitutes the core mechanosensing machinery of cadherin adhesions.**R. Seddiki¹, B.R. Sarangi², G. Peyret², E. N'Guyen³, R. Mege², B. Ladoux^{2,3};¹Institut Jacques Monod, University Paris Diderot CNRS, Paris, France, ²Institut Jacques Monod, Université Paris Diderot CNRS, Paris, France, ³Mechanobiology Institute, National University of Singapore, Singapore, Singapore

Tissue-scale mechanics is important in morphogenesis, homeostasis and wound healing. Cells exert mechanical force on each other at sites of cell-cell adhesion, typically through cadherins. We demonstrated previously that cadherin adhesions transmit and adapt to mechanical stress. Here we aim at understanding how mechanical stress regulates the formation and strengthening of cell-cell contacts and at identifying the molecular mechanosensor involved. We focus here on the role of α -catenin which links cadherins to the acto-myosin network. We recently demonstrated that α -catenin reversibly unfolds upon physiologically-relevant forces allowing the binding of another actin binding protein, vinculin (Yao et al, Nature Communication, 2014), suggesting that the tension-dependent binding of vinculin to α -catenin is essential for tension-dependent cell-cell contact strengthening. By plating epithelial MDCK cells on micro-patterned, fibronectin-coated, substrates of controlled rigidities, we first showed that the stability of E-cadherin and α -catenin at cell-cell contacts, as analyzed by FRAP, as well as the extent of recruitment of vinculin and actin increases with stiffness. We then generated GFP-tagged mutated α -catenin constructs: one bearing a point mutation in the vinculin binding α -helix (L344P) and the other deleted of the MI and MII domains masking the vinculin binding domain (Δ mod), not able to bind and constitutively binding vinculin, respectively. Both constructs expressed in α -catenin-depleted MDCK cells restored the formation of E-cadherin-mediated cell-cell contacts, although L344P did not allow vinculin recruitment. We further show that the binding of vinculin regulates the stiffness-dependent stabilization of α -catenin at cell-cell contacts. The analysis of cell-cell cohesion dynamics, by Particle Imaging Velocimetry, and force transmission, by micro-force sensor array, of mutants and wt α -catenin expressing cells plated on confined fibronectin-coated substrates revealed that cell-cell contact stability, cell-to-cell mechanical coupling and collective movement correlation length increase from L344P to wt and Δ mod, i.e. with the ability of α -catenin to bind vinculin. Thus the interaction between α -catenin and vinculin is crucial for cells to develop stable cell-cell contacts and long-range interactions as well as coordinated motions. The fact that the association between the two proteins, manipulated here by mutagenesis, has a direct incidence on adhesion complex stability and complex collective cell behavior put this interaction at the center of the cadherin mechanosensing machinery. Further studies will allow to decipher how this interaction stabilizes α -catenin and the recruitment of the acto-myosin network in force-dependent manner.

P602**Board Number: B853****Alpha-T-catenin is constitutively-active actin-binding protein that couples the cadherin complex to the actin cytoskeleton.**A.V. Kwiatkowski¹, E. Wickline¹, I. Dale¹, C. Merkel¹;¹Cell Biology, Univ Pittsburgh Sch Med, Pittsburgh, PA

The core component of the adherens junction, the cadherin-catenin complex, organizes the actin cytoskeleton to regulate cell-cell adhesion and tissue organization in metazoans. The primary link between the cadherin-catenin complex and actin is α -catenin, a multifunctional actin-binding protein that regulates cell adhesion, movement and signaling. Mammals possess three α -catenins – α E(Epithelial), α N(Neural) and α T(Testes)-catenin. In contrast to the widely studied and well-defined mammalian α E-catenin, little is known about the molecular properties of α T-catenin, a protein unique to amniotes and expressed predominantly in cardiomyocytes where it localizes to cell-cell junctions. Notably, mutations in α T-catenin have been linked to arrhythmogenic right ventricular cardiomyopathy (ARVC). However, it is not clear how these mutations affect the biochemical properties of α T-catenin or impact α T-catenin functions in cardiomyocyte adhesion. We hypothesized that α T-catenin is specialized to meet the demands of cardiomyocyte adhesion. To test this, we analyzed the molecular properties of mouse α T-catenin and found that it has key functional differences from mouse α E-catenin. Size exclusion chromatography and native PAGE revealed that α T-catenin is primarily a monomer in solution, unlike α E- and α N-catenin that can readily form homodimers. α T-catenin monomer bound to filamentous actin with K_d of 0.2 μ M in cosedimentation assays, roughly twice as strong as α E-catenin homodimer (0.5 μ M). The β -catenin/ α T-catenin heterocomplex bound to F-actin with a K_d of 0.7 μ M. Addition of β -catenin lowers the affinity of α T-catenin for F-actin only slightly, unlike the dramatic decrease in F-actin binding observed with the β -catenin/ α E-catenin complex (>10 μ M). Finally, we examined how a mutation in α T-catenin linked to ARVC – V94D – affected protein function. Strikingly, the α T-catenin V94D mutant protein eluted as a homodimer in size exclusion chromatography and failed to bind β -catenin in pulldown assays. Thus, the V94D mutation creates an α T-catenin homodimer that cannot bind β -catenin, and we predict this mutation impairs α T-catenin function in cardiomyocytes. Our data demonstrate that α T-catenin is a constitutively-active actin-binding protein that can functionally couple the cadherin-catenin complex to F-actin, properties that make it unique from mammalian α E-catenin. These studies begin to define the molecular mechanisms underlying α T-catenin function in cell-cell adhesion and how disruptions in α T-catenin properties can lead to cardiomyopathies.

P603**Board Number: B854****Arp2/3 mediated membrane activity induces membrane fusion at self-contacts.**G. Sumida¹, S. Yamada¹;¹Biomedical Engineering, Univ California-Davis, Davis, CA

Actin assembly is required for membrane activities that drive cell adhesion and migration. The Arp2/3 complex, a potent actin nucleator, binds to the side of actin filaments and recruits additional actin monomers to generate a new branching actin filament. While Arp2/3 complex-mediated actin polymerization plays a critical role in membrane dynamics at the leading edge of migrating cells, the roles of the Arp2/3 complex is not limited to cell adhesion and migration. Interestingly, recent studies implicate the roles of actin dynamics in cell-to-cell fusion. Using micron-sized obstacles to induce the formation of self-contacts in epithelial cells, we showed that the efficiency of such self-adhesion depends on E-cadherin, a calcium dependent cell-cell adhesion molecule, but is distinct from cell-to-cell adhesion in that self-contacts are eliminated by membrane fusion. At self-contacts, while the concentration of E-cadherin diminished, the intensity of GFP-tagged Arp3 rapidly fluctuated, then decreased and stabilized after membrane fusion. Furthermore, the Arp2/3 complex inhibitor prevented Arp3 from localizing near self-contacts and reduced self-contact induced membrane fusion. We identified Rac1 as a potential upstream regulator of the Arp2/3 complex in membrane fusion, as both dominant-negative Rac1 and a Rac1-specific inhibitor decreased membrane fusion at self-contacts. Taken together, these data suggest that the Arp2/3 complex induced actin polymerization likely brings two opposing membranes into close apposition by excluding E-cadherin, thus promoting membrane fusion at self-contacts. Our analysis extends the roles of actin dynamics in self-contact induced membrane fusion, and provides new insights into the elimination of self-junctions during phagocytosis or the formation of seamless, single-cell capillaries.

P604**Board Number: B855****Dual role of Src in regulation of endothelial barrier.**J.E. Brinks Klomp¹, M. Patel¹, A.V. Karginov¹;¹Pharmacology, University of Illinois - Chicago, Chicago, IL

Adherens junctions (AJs) mediate cell-cell interactions, crucial for regulating endothelial cell permeability. The role of Src tyrosine kinase in the regulation of AJs is controversial. It can stimulate AJ disassembly, but its activity is required for AJ formation, suggesting a dual function of Src in regulation of endothelial barrier. We hypothesized that the specific effect of Src on AJ depends on the duration and the level of kinase activity. To test this we employed a recently developed technology for engineered activation of Src that provides specificity and tight temporal control of kinase activity in living cells. Our studies demonstrate that Src activation causes a distinct biphasic temporal effect on endothelial permeability. Initially Src induction leads to strengthening of endothelial barrier and then later increases

barrier permeability. This suggests that sustained elevated Src activity increase endothelial permeability, whereas transient activation of Src promotes barrier function. To gain insight into the mechanism of these effects we analyzed how Src activation affects phosphorylation and interactions of AJ proteins (VE-cadherin, β -catenin and p120-catenin), and AJ morphology. Src activation was sufficient to induce phosphorylation of VE-cadherin at the early time points, but it did not disrupt VE-cadherin association with catenins. Following Src activation VE-cadherin initially stayed in the cell-cell junctions, but underwent morphological rearrangement. Only at the later time points it was internalized to the perinuclear region. Src activation also altered the arrangement of F-actin. F-actin initially formed actin foci and was enriched within lamellipodia following Src activation. However, at later times F-actin became arranged in centripetal actin bundles at the edge of the cell. Interestingly, the morphological changes in AJs, VE-cadherin re-localization and F-actin reorganization temporally correlated with Src-induced biphasic changes in endothelial permeability. Our studies also show that the Src-mediated changes associate with an increase in Rac1 GTPase activation, suggesting that Rac1 activation is possibly the driving force for the temporal changes in VE-cadherin localization and F-actin arrangement which in turn alter endothelial cell permeability. Currently, we are expanding the inducible kinase system to other SFKs and analyzing their role in regulation of endothelial permeability.

P605

Board Number: B856

dGirdin supports cell-cell adhesion and maintains epithelial tissue integrity.

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Introduction: The human Girdin protein is overexpressed in various cancers, and promotes cell migration and invasion. This suggests that Girdin contributes to tumor progression. Recently, it was shown that Girdin directly interacts with Par-3. This interaction is essential for cell polarization associated with directed cell migration. Par-3 and its Drosophila ortholog Bazooka (Baz) are also known for their role in the establishment and maintenance of epithelial cell polarity and adherens junction formation. Epithelial polarity, which is characterized by the asymmetric distribution of many cellular constituents, is necessary for epithelial tissue function and homeostasis. Indeed, loss of several epithelial polarity regulators leads to epithelial to mesenchymal transition (EMT).

Hypothesis: We hypothesized that Girdin plays a role in epithelial polarity and/or cell-cell adhesion, as reported for its binding partner Par-3. In order to test this premise *in vivo*, we generated null alleles of *dgirdin* (*dgir*), which encodes the Drosophila ortholog of Girdin, and established specific anti-dGir antibodies.

Results: First, we demonstrated that dGir is mainly expressed during embryogenesis. In embryonic epithelial cells, it is predominantly associated with the plasma membrane and enriched at adherens junctions. Besides, we found that phosphorylation of dGir increases its association with the

cytoskeleton. *dgir* mutant embryos show dorsal closure failure caused by impaired collective cell migration. Thus, dGir is important for cell motility, as reported for its mammalian counterpart. We also observed that some cells detach from the ectoderm in the absence of dGir. This individualization of epithelial cells is sometimes accompanied by an opening of the ventral midline, suggesting that loss of dGir weakens cell-cell adhesion. Consistent with this phenotype, the association between adherens junction components, including Armadillo (β -catenin) and DE-Cadherin, and the cytoskeleton decreases in *dgir* mutant animals. These results suggest that dGir participates in junction stability by favouring attachment to the F-Actin network. Finally, we noticed the presence of ectopic epithelial cell cysts in *dgir* mutant embryos. These cysts originate from ectopic invagination of ectodermal cells. Cyst cells show expansion of the apical membrane, indicating polarity defects.

Conclusion: Together, our data identify dGir as a novel regulator of epithelial cell polarity and cell-cell adhesion. These results thus reveal unsuspected roles for Girdin related proteins. Comprehensive analysis of Girdin/dGir function is essential to evaluate whether it is an appropriate target to treat cancer.

P606

Board Number: B857

ARF6 activation in response to Wnt stimulation leads to increased endocytosis and mitotic spindle defects in epithelial cells.

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Regulation of surface proteins, from growth factor receptors to cell adhesion components, is critical to the development and maintenance of epithelial tissues. ADP-ribosylation factor 6 (ARF6) is a member of the Ras-related GTPases with well-documented roles in controlling endocytosis of cell surface components. For example, stimulation of epithelial cells with hepatocyte growth factor leads to the activation of ARF6 and subsequent internalization of both c-Met (the HGF receptor) and E-cadherin. Here we demonstrate that ARF6 activation during canonical Wnt signaling leads to the intracellular accumulation of β -catenin, c-Met and E-cadherin in endosomal compartments along with robust activation of the extracellular signal related kinase (ERK). Disassembly and endocytosis of adherens junctions is dependent on Wnt induced ERK activation, as treatment with the MEK inhibitor PD98059 blocks the internalization of E-cadherin while having no effect on the accumulation of internal pools of c-Met. These findings suggest that Wnt-induced endocytosis of c-Met and E-cadherin likely occurs via distinct pathways. Wnt stimulation also leads to a defect in mitotic spindle orientation with cells dividing in a manner that leaves one daughter cell growing out of the epithelial monolayer. Misorientation of the mitotic spindle appears to be independent of ERK activation and E-cadherin endocytosis. Thus, canonical Wnt signaling can effect multiple cellular changes which have profound impact on epithelial architecture.

P607**Board Number: B858****Functional E-cadherin is required for translation-dependent beta-actin cytoskeleton remodeling at epithelial cell-cell contact sites.**L.A. Cruz¹, A.J. Rodriguez¹;¹Federated Department of Biology, Rutgers University, Newark, NJ

Epithelial adherens junction complexes are composed of a single pass transmembrane glycoprotein, E-cadherin, and juxta-membrane localized catenin family members (ρ -120, β - and α -catenin) that anchor to the actin cytoskeleton. In addition, adherens junction complexes are prominent sites for regulated phosphorylation and dephosphorylation events including Src activation by E-cadherin homophilic binding. Moreover, E-cadherin homophilic binding activates RhoA to localize β -actin mRNA at cell-cell contacts sites. The active Src at cell-cell contacts then releases the translational repression of the β -actin mRNA allowing localized β -actin monomer synthesis at cell-cell contact sites driving the anchoring of the adherens junction complex to the actin cytoskeleton during adherens junction maturation. Using Madin-Darby Canine Kidney (MDCK) cells and E-cadherin function blocking antibodies we demonstrate E-cadherin homophilic binding regulates spatially localized β -actin monomer synthesis during de novo adherens junction assembly in Ca^{2+} switch experiments using the β -actin translation site imaging method. The effects of blocking E-cadherin homophilic binding were quantified as the ratio of colocalization between adherens junction markers and F-actin at the cell periphery to that in the cytoplasm. Preliminary experiments using function blocking antibodies confirm blocking E-cadherin homophilic binding perturbs de novo adherens junction assembly. In addition, assembly of circumferential actin belts 3-hours post-contact are perturbed characterized by radial arrays of F-actin at cell-cell contacts sites in E-cadherin blocking antibody treated cells. We hypothesize E-cadherin homophilic binding activates Src at cell contacts that in turn is required to initiate β -actin mRNA translation. β -actin translation site imaging studies support this hypothesis because β -actin translation sites appear to be present throughout the whole cell with a decrease in β -actin synthesis observed at cell contacts in E-cadherin blocking antibody treated cells. These results demonstrate E-cadherin homophilic binding is required for contact localized β -actin translation, which is required to remodel the actin cytoskeleton providing linear filaments used to anchor and stabilize adherens junction complexes at epithelial cell-cell contact sites.

P608**Board Number: B859****Size Exclusion Chromatographic Assay of Dimerization by Epithelial Cadherin.**C.S. Fox¹, S.D. Pedigo²;¹Chemistry and Biochemistry, University of Mississippi, Oxford, MS, ²Chemistry and Biochemistry, The University of Mississippi, Oxford, MS

The classical cadherin family is an extensively studied family of calcium- dependent homophilic cell adhesion proteins, which includes epithelial cadherin (ECAD) and neural cadherin (NCAD). ECAD plays essential roles in the formation and maintenance of epithelial tissues. The adhesive interface lies in the first of five similar tandemly repeated extracellular domains (EC1-EC5) and requires exchange of the N-terminal A-strand. The purpose of the experiments presented here is to develop a simple assay for dimerization using purified recombinant protein. A chromatographic assay has been developed for NCAD, which essentially involves trapping the protein in a dimeric state thus significantly reducing the exchange rate between states. This allows resolution of monomer and dimer peaks using size exclusion chromatography. No equivalent assay for the study of ECAD dimerization has been established until now. The K14E mutant of ECAD experiences slow exchange between monomer and dimer, although the dimerization affinity is unchanged by the mutation. Studies presented here establish a chromatographic assay for ECAD dimerization affinity using the K14E mutant. Control experiments demonstrate that equilibration requires 4 hours and dimerization occurs in a calcium and protein dependent manner. This assay is useful for determining the effect of microenvironmental factors that will influence dimerization by ECAD in vivo. NSF MCB 0950494 and DOEd GAANN P200A120046-13

P609**Board Number: B860****Nickel reduces calcium-dependent dimerization by Neural Cadherin.**M. Dukes¹, R.K. Rowe¹, S.D. Pedigo¹;¹Chemistry and Biochemistry, The University of Mississippi, Oxford, MS

Cadherins are the primary transmembrane component in adherens junctions, structures that link the actin cytoskeleton in adjacent cells within tissue. Adherens junctions occur in most solid tissues including neurological synapses, epithelium and endothelium. Cell-cell adhesion by cadherins requires the binding of calcium ions to specific sites in the extracellular domain. Requirement of calcium binding beckons the question of whether other divalent cations might substitute for or compete with calcium for site occupancy. The studies reported herein characterize the biophysics of nickel binding to neural cadherin. Based on results from thermal-denaturation studies, it was clear that neural cadherin was stabilized by the presence of nickel in solution. Furthermore, we attempted to determine the K_d for nickel binding by direct titrations of neural cadherin monitored by circular dichroism. The K_d was unable to be determined due to the absence of a saturated baseline. Subsequent experiments tested whether the presence of constant low levels of nickel affected calcium binding affinity and calcium-induced

dimerization. Interestingly, there was a significant effect upon the apparent calcium binding affinity and on adhesive dimer formation as a function of nickel concentration, suggesting that detrimental competition for site occupancy occurs between these divalent cations.

P610

Board Number: B861

An assay to study cellular responses to simultaneous mechanical and chemical gradients.

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Cell response to chemical cues is at the core of a myriad of fundamental biological processes that range from angiogenesis to cancer metastasis. Most of these biological processes are also influenced by mechanical cues from the extracellular matrix. How chemical and mechanical cues act in synergy to drive biological function is unknown, however. Here we present a robust microfluidic device that combines the precise diffusion of chemical factors together with well-defined stiffness profiles. The device generates a stable, linear and purely diffusive chemical gradient over a biocompatible hydrogel with a well-defined and perpendicular stiffness gradient. Device fabrication relies on a recently developed technique based on patterned PSA (Pressure Sensitive Adhesive) double side tape stacks that can be implemented with minimal cost and lab equipment. This technique is suitable for long-term observation of cell migration and the application of traction force microscopy. We validate our device by testing MDCK cell scattering in response to perpendicular gradients of HGF and substrate stiffness.

P611

Board Number: B862

FilGAP, a Rho/ROCK-regulated GAP for Rac controls adherens junctions in MDCK cells.

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Cell-cell adhesion is essential for epithelial tissue integrity and functions. Adherens junctions (AJs) are responsible for epithelial cell-cell adhesions and are composed of a transmembrane protein E-cadherin that is connected to actin cytoskeleton via catenins. Rho family small GTPases are essential for the formation of adherens junctions of epithelial cells. We found FilGAP, a Rac specific Rho GTPase-activating protein, promotes the formation of adherens junctions of Madin-Darby canine kidney (MDCK) cells. Knockdown of FilGAP by siRNA stimulated the disassembly and migration of MDCK cells induced by Hepatocyte-growth factor (HGF). Conversely, forced expression of FilGAP induced accumulation of E-

cadherin at adherens junction and suppressed HGF-induced scattering. Reduction of medium Ca^{2+} disrupted the cell-cell adhesion and restoration of Ca^{2+} re-established the cell-cell adhesion. Depletion of FilGAP by siRNA prevented cell-cell adhesion induced by Ca^{2+} . The Rac GAP domain of FilGAP is necessary for suppression of scattering. In agreement with this observation, knockdown of endogenous Rac by siRNA suppressed disassembly of MDCK cells induced by HGF. Forced expression of Rho kinase (ROCK) induced accumulation of E-cadherin at adherence junction and depletion of FilGAP by siRNA prevented the accumulation of E-cadherin. Moreover, phospho-mimic FilGAP mutant but not non-phosphorylatable FilGAP mutant stimulated accumulation of E-cadherin at cell junctions. These results suggest that FilGAP may regulate cell-cell adhesion through inactivation of Rac downstream of Rho/ROCK-signaling in MDCK cells.

P612

Board Number: B863

Histatin 1, a histidine-rich peptide in human saliva, promotes cell adhesion and plays a role in mesenchymal-epithelial transition.

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Histatins are histidine-rich peptides produced by the salivary glands of higher primates. This study explores the effects of histatins on cell-substrate adhesion and cell-cell adhesion of epithelial cells and fibroblasts.

We found that single cells seeded in serum-free medium attach and spread significantly faster in the presence of histatin 1 (Hst1). This effect was specific for Hst1, since other tested histatins did not stimulate cell-substrate adhesion. Hst1 causes a more than 2-fold increase in the ability of cells to attach and spread, even under conditions that impair cell adhesion. Rapid cell-substrate adhesion is particularly important for junctional epithelium, an intermediate cell type between gingival tissue and tooth enamel acting as a protective seal against microbial and physical stresses.

The effect of Hst1 on cell-cell adhesion was investigated using trans-epithelial resistance (TER) measurements in Caco-2 cells, a widely used model system for the epithelial layer. Hst1 causes an over 20% increase in TER compared to control cells. A role for Hst1 in both cell-substrate and cell-cell adhesion is highly conceivable, because these two modes of adhesion are closely related via connected signaling pathways and shared components.

Using confocal microscopy we observed that Hst1 induced a more than 2-fold increase in the amount of active E-cadherin, a cell-cell adhesion protein, and a 50% increase in the amount of the tight junction protein ZO-1 in Caco-2 cells. Our results indicate that Hst1 is involved in maintaining the epithelial architecture and barrier function. This barrier is not only important in the mouth, which is a challenging environment where food, heat, cold and acidic compounds all induce stress to the soft tissues of the

oral cavity, but also for the total of epithelial layer covering the cavities and surfaces throughout the body.

E-cadherin activation is recognized as a hallmark for mesenchymal-epithelial transition (MET), a process important during embryogenesis, development, cell differentiation and cancer metastasis.

Immunochemical staining of Hst1 revealed the presence of Hst1 in several layers of the oral epithelium of 16-20 weeks old fetuses. Hst1 was not present in fetal salivary glands, which are not yet functional at this age, indicating that oral epithelial cells produce Hst1 during fetal development. Together our results suggest that Hst1 promotes MET.

P613

Board Number: B864

VE-cadherin is proteolytically processed upon endocytosis to remove the beta-catenin binding domain.

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Regulation of endothelial cell adhesion is essential in many biological processes including wound healing, angiogenesis, inflammation and cancer metastasis. VE-cadherin trafficking to and from the plasma membrane has emerged as a critical mechanism for regulating surface levels of VE-cadherin and adherens junction adhesion strength. Despite the fact that VE-cadherin trafficking has been under extensive investigation, little is known about the role of proteolytic processing in regulating VE-cadherin trafficking. To investigate this process further, endothelial cells were treated with chloroquine to inhibit lysosomal degradation. This treatment resulted in the accumulation of a 95kD VE-cadherin fragment, suggesting that VE-cadherin is cleaved during the process of internalization and trafficking to endosomal compartments. Using biochemical and fluorescent imaging of internalized and surface pools of cadherin, we found that the b-catenin binding domain of VE-cadherin is removed upon internalization. Further analysis suggests that this cleavage occurs between the juxtamembrane domain and the catenin-binding domain of the VE-cad tail. To determine if endocytosis is required for cleavage, we utilized pharmacological inhibitors of clathrin-mediated endocytosis as well as VE-cadherin point mutants resistant to internalization. Both of these approaches indicated that endocytosis of the cadherin is required for proteolytic processing. Furthermore, over-expression of p120-catenin, which inhibits VE-cad endocytosis, blocked VE-cadherin cleavage. These findings suggest that VE-cadherin is proteolytically processed upon endocytosis to remove the beta catenin binding domain, raising the possibility that this cleavage event is critical in regulating both VE-cadherin trafficking and adhesive function.

P614**Board Number: B865****Quantitative Real Time Imaging of Protein Secretions from Single Cells.**M.P. Raphael¹, J.A. Christodoulides¹, J.B. Delehanty¹, J.P. Long¹, J.M. Byers¹;¹Naval Research Laboratory, Washington, DC

Cell-to-cell signaling often involves the secretion of proteins, which creates spatial and temporal concentration profiles in the extra-cellular environment for the receiving cells to detect and interpret. Real-time measurements of secreted protein concentrations at the single cell level are thus fundamental to understanding these communications pathways but have proven difficult to realize in practice. Here we present a label-free technique based upon nanoplasmonic imaging and high-affinity binding which enabled the measurement of individual cell secretions in real time. When applied to the detection of antibody secretions from individual hybridoma cells, the enhanced time resolution revealed two modes of secretion: one in which the cell secreted continuously and another in which antibodies were released in concentrated bursts that coincided with minute-long morphological contractions of the cell. From the continuous secretion measurements we determined the local concentration of antibodies at the sensing array closest to the cell. The technique is incorporated on to a wide-field microscope which enables real-time transmitted light and fluorescence imaging of the cells as well. We anticipate this technique will be broadly applicable to the real-time characterization of both paracrine and autocrine signaling pathways.

Structure and Function of the Extracellular Matrix**P615****Board Number: B866****Hydraulic fracture during epithelial stretching.**L. Casares Garcia^{1,2}, R. Vincent¹, D. Zalbidea¹, N. Campillo², D. Navajas^{1,2,3}, M. Arroyo⁴, X. Trepats^{1,2,5};¹Institute for Bioengineering of Catalonia (IBEC), Barcelona, Spain, ²University of Barcelona, Barcelona, Spain, ³Ciber Enfermedades Respiratorias, Bunyola, Spain, ⁴LaCàN, Universitat Politècnica de Catalunya-BarcelonaTech, Barcelona, Spain, ⁵Institució Catalana de Recerca i Estudis Avançats (ICREA),, Barcelona, Spain

The epithelium is a cohesive cell sheet adhered on a soft hydrogel matrix that covers free surfaces and cavities throughout the body. During development and adult life, the epithelium performs a broad diversity of functions including morphogenesis, wound healing, tissue compartmentalization, and protection against environmental pathogens. Epithelial sheets carry out these functions in a dynamic mechanical environment characterized by elevated levels of cell and tissue stretching. Failure to withstand stretching leads to epithelial fracture, which is associated with impaired development and severe pathological conditions. Epithelial fracture is commonly attributed to excessive tension in key stress bearing elements of the epithelium such as the cytoskeleton, the plasma membrane, and cell-cell

junctions. We developed a new experimental approach to study fracture dynamics of micropatterned epithelial monolayers adhered to soft hydrogel substrates. Using this approach, we demonstrate epithelial cracks caused by tissue stretching but independent of epithelial tension. We show that the origin of these cracks is hydraulic; they form to release a transient buildup of pressure in the hydrogel substrate during stretch/unstretch maneuvers. Shortly after stretch cessation, transepithelial pressure equilibrates and cracks seal through an acto-myosin dependent mechanism. We demonstrate this behavior in a variety of synthetic and physiological hydrogel substrates including polyacrylamide, matrigel, and decellularized animal tissue. The theory of poroelasticity successfully captures the observed phenomenology and allows us to predict the size and healing dynamics of epithelial cracks as a function of the stiffness, geometry, and composition of the hydrogel substrate. Thus, coupling between tissue stretching and matrix hydraulics determines epithelial integrity in a tension-independent manner.

P616

Board Number: B867

Extracellular Matrix Remodeling Regulates Age-Associated Cardiomyocyte Function.

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Increased deposition of extracellular matrix (ECM) is observed in all advanced age heart failure patients, but current model systems are complex and slow to age. To investigate the effect of extracellular remodeling on mechanical function in genetically tractable, rapidly aging, and simple model organisms, we employed *Drosophila melanogaster*, which has a bilayered heart tube. We found that two common wildtype strains of *Drosophila*, i.e. *yellow-white (yw)* and *white-1118 (w¹¹¹⁸)*, exhibit different cytoskeletal and ECM remodeling with age. Using a recently developed nanoindentation method to measure cardiomyocyte stiffness and high speed optical imaging to assess contractility of intact *Drosophila* hearts, we found that *yw* flies had stiffer intercalated discs (ICD) and exhibited diastolic dysfunction with age. On the other hand, *w¹¹¹⁸* flies did not exhibit ICD stiffening, exhibited a less severe diastolic dysfunction, and showed an increase in ECM layer thickness between ventral muscle (VM) and cardiomyocyte (CM) layers of the heart tube. To modulate ECM and assess its effect in the aged *w¹¹¹⁸* flies, we knocked-down ECM genes LamininA (Laminin 3,5) and Viking (Collagen IV) and found no effect on cardiac performance in juvenile flies, but in aged flies, it decreased cardiomyocyte stiffness and increased contractile irregularity. This suggests that the cell-ECM contacts in the basement membrane are intimately tied not only to the coupling of the cardiomyocytes of the *Drosophila* heart tube but also to cytoskeletal remodeling. These data may also have larger implications for elderly patients suffering from extensive age-related myocardial remodeling and fibrosis who experience cardiomyocyte decoupling and resultant arrhythmias.

P617**Board Number: B868****Synergistic activation of fibroblasts by a modest increase in stiffness in combination with serum cytokines in a 3-D model of the tumor stroma.**J.S. McLane¹, L.A. Ligon¹;¹Biology, Rensselaer Polytechnic Inst, Troy, NY

Activated fibroblasts have been shown to enhance the progression of metastatic disease. These cancer-associated fibroblasts are found in the transformed extracellular matrix (ECM) that surrounds tumors such as those of the mammary gland. One important property of the transformed ECM is an approximately ~3-4 fold increase in stiffness in comparison to normal mammary tissue. This increase in stiffness is conserved among multiple tumor types, leading us to hypothesize that differences in 3-D matrix stiffness can cause fibroblast activation. To test this, we developed an in vitro model in which fibroblasts were grown in a collagen-I based 3-D hydrogel system where the stiffness is controlled independently of collagen concentration by chemical crosslinking. We generated collagen-I hydrogels representing both normal breast stroma (~200 Pa) and tumor associated stroma (~800 Pa). Our results indicate that this modest (~600 Pa), but physiologically relevant, increase in matrix stiffness, leads to a partial activation of fibroblasts toward a myofibroblast phenotype, but that full activation requires the contribution of serum factors. We conclude, therefore, that the activation of fibroblasts in the tumor microenvironment involves the synergistic activity of increased matrix stiffness and chemokine signaling.

P618**Board Number: B869****Interplay of Extracellular Matrix Stiffness and Tethering in Regulating Stem Cell Fate.**J.H. Wen¹, L.G. Vincent¹, A. Fuhrmann¹, Y. Choi², K.C. Hribar³, H. Taylor-Weiner¹, S. Chen³, A.J. Engler¹;¹Bioengineering, University of California, San Diego, La Jolla, CA, ²Kolling Institute of Medical Research, Sydney, Australia, ³Nanoengineering, University of California, San Diego, La Jolla, CA

Stem cells “feel” and differentiate in response to the surrounding extracellular matrix (ECM) properties integrating signals such as stiffness and ligand type, by binding to and contracting against the matrix. Recently, the degree of fibrous protein coupling to the surface of the underlying substrate, i.e., tethering and matrix porosity, has also been proposed to mechanically regulate differentiation. In order to further investigate how these factors and substrate mechanics contribute to cell fate, we modulated substrate stiffness, porosity, and surface ligand tethering density of polyacrylamide hydrogels to investigate how these factors collectively or independently regulate cell fate. Varying substrate porosity while maintaining constant stiffness did not significantly change collagen protein tethering observed by atomic force microscopy (AFM), substrate deformations observed by traction force microscope (TFM), or osteogenic and adipogenic differentiation of sub-confluent human adipose-derived stromal cells (ASCs)

or marrow-derived mesenchymal stem cells. Adjusting protein-substrate linker sulfo-SANPAH density while maintaining constant porosity changed tethering, but did not affect osteogenesis or adipogenesis, surface protein unfolding by cells detected via FRET, and underlying substrate deformations determined via TFM. To eliminate the possibility of surface protein deformations altogether, the short cell-adhesive peptide RGD was incorporated into the substrate; differentiation of ASCs and MSCs was unaffected by the absence of fibrous protein tethering or peptide density as only softer substrates prevented sub-confluent osteogenesis, and stiffer substrates prevented adipogenesis. Careful investigation of the elastic mechanical properties of PDMS and polyacrylamide using AFM further supports that these conclusions extend to other systems. Together, our data imply that cell-generated deformations of planar matrices of a specific stiffness regulate stem cell differentiation independently of protein tethering and porosity.

P619

Board Number: B870

IL-1 β promotes mesenchymal stem cell migration by inducing the expression of MMP-1.

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Mesenchymal stem cells are known to “homing” to areas of injury in response to signals of cellular damage. However, the characteristics, mechanisms, and significance of how cytokines can directly influence the stem cells and recruit it to the target tissue are still unclear. In this study, we found that the pro-inflammation cytokine interleukin-1 β (IL-1 β) promotes mesenchymal stem cells migration. The cDNA microarray data shows that IL-1 β induces matrix metalloproteinase-1 (MMP-1) expression. We then used quantitative real-time PCR and MMP-1 ELISA to verify the results. Pre-treatment with IL-1 β inhibitor interleukin-1 receptor antagonist, MMP-1 inhibitor tissue inhibitor of metalloproteinases, and protease activated receptor 1 inhibitor SCH79797, the results showed that PAR1 protein signaling pathway leads to cell migration. In conclusion, IL-1 β promotes the secretion of MMP-1 which then activate the PAR1 and by a G-protein-coupled signal pathway to promote mesenchymal stem cells migration.

P620**Board Number: B871****ECM Geometry Promotes Directed Cell Migration by Regulating Leading Edge Dynamics.**G.R. Ramirez-SanJuan¹, P.W. Oakes², M.L. Gardel²;¹James Frank Institute, Institute for Biophysical Dynamics and Physics Department, University of Chicago, Chicago, IL, ²James Franck Institute, Institute for Biophysical Dynamics and Physics Department, University of Chicago, Chicago, IL

During development and tissue morphogenesis dynamic changes in cell motility and shape can be regulated by extracellular matrix (ECM) physical cues. Fibrillar ECM geometries are a hallmark of several developmental processes, however *in vivo* it is difficult to assess the influence of ECM geometry in directing cell migration and promoting tissue rearrangements. Furthermore, the physical parameters and cytoskeletal organelles that control geometry sensing in cells are unknown. Here we use UV micropatterning to mimic aligned ECM fibrils and study the influence of subcellular changes in ECM geometry in 3T3 cell shape and migration. We pattern 2 μ m thick Fibronectin parallel lines onto an elastic substrate, varying the spacing between lines at a subcellular lengthscale (0-10 μ m). On uniformly coated substrates, cells migrate in a random direction, however in linear geometries cells acquire an elongated morphology and migrate preferentially along the direction of the ECM lines. As we increase the spacing between lines, we find that migration directionality and shape elongation increase monotonically as a function of distance between lines. Since the shape of a migrating cell is regulated by its protrusive and contractile organelles, we investigated their contribution to ECM geometry sensing. We find that inhibition of Myosin II has a modest effect on the ability of cells to sense geometry changes. In contrast, upregulation of lamellipodial protrusion, by expression of a constitutively active form of Rac, impairs the ability of cells to migrate along axis of the ECM lines. Further analysis of the protrusive dynamics in cells migrating on uniform and linear substrates reveals that lamellipodial protrusions are concentrated in the direction of the ECM axis in linear substrates. These protrusions are longer lived as compared to protrusions perpendicular to the ECM axis, thus biasing the direction of cell migration and promoting an elongated cell morphology. Finally, we tested the relevance of ECM geometry sensing in individual cells in a multicellular context. To this end we grew MDCK cell monolayers on micropatterns with linear ECM geometries, similar to single cells, in these monolayers cells elongate and exhibit directional motion in line spacings as small as 2 μ m. Thus we propose a model where ECM fibrillar geometries influence the direction of cell migration in a multicellular assembly, by acting at an individual cell level to increase the persistence of protrusive activity along the axis of the fibrils. Our work demonstrates that ECM geometry can provide an asymmetry that influences migration dynamics both in single cells and multicellular assemblies, highlighting the importance of physical properties of the ECM for development and tissue morphogenesis.

P621**Board Number: B872****Induction of Kidney Fibrosis by Fibronectin Synthesis via WNT Signaling.**A. Kuma¹, M. . Tamura², S. Yamada³, H. Ueno¹, N. Ishimatsu¹, Y. Fujimoto¹, K. Bando², R. Serino², Y. Otsuji¹, K. Kohno⁴;¹Second Department of Internal Medicine, University of Occupational and Environmental Health, Kitakyushu, Japan, ²Kidney Center, University Hospital of Occupational and Environmental Health, Kitakyushu, Japan, ³Department of Pathology and Cell Biology, University of Occupational and Environmental Health, Kitakyushu, Japan, ⁴The President Laboratory, University of Occupational and Environmental Health, Kitakyushu, Japan

Background: Acute interstitial nephritis (AIN) induces kidney fibrosis after acute kidney injury (AKI). We previously reported WNT10A as a novel angio/stromagenic factor useful in wound healing. However, the relationship between WNT10A and kidney fibrosis is not completely clear. Here we investigated the role of WNT10A in the fibrotic process in AIN. **Methods:** Kidney samples from 20 male patients (≥ 60 years) diagnosed with AIN by kidney biopsy at our hospital were analyzed by immunohistochemistry (IHC) for WNT proteins, α -SMA, and fibronectin. The relationship between estimated glomerular filtration rate (eGFR) and WNT protein expression was evaluated. COS-1 cells (obtained from kidney fibroblasts of African green monkey) were transfected with a WNT10A expression plasmid (WNT10A overexpressing cells; COS-1-10A). Protein expressions of cell lysates were detected by western analysis and cytotoxicity was analyzed by WST-8 assay. **Results:** Of the 20 patients, 10 with WNT10A expression in biopsy tissue had significantly lower eGFR values (median, 11.12 mL/min per 1.73 m²) than the remaining 10 with no detectable WNT10A expression (median, 34.70; $p=0.0002$). The relationship between eGFR and the expression level of any other WNT protein examined (WNT-1, -3, and -4) was not significant. IHC WNT10A positive samples showed a positive correlation with fibronectin positive ($p = 0.0062$) and α -SMA-positive ($p = 0.0005$) samples. Furthermore, compared with control COS-1 cells, COS-1-10A enhanced the expression of fibronectin around 3- to 5-fold. COS-1 10A increased the level of peroxiredoxin (PRDX) 5 expression and rendered fibroblasts resistant to hydrogen peroxide (15-62.5 μ M). However, downregulation of PRDX 5 expression by siRNA sensitized COS 1 10A cells to hydrogen peroxide. **Conclusion:** WNT10A expression may promote kidney fibrosis following severe kidney dysfunction. WNT10A-expressing fibroblasts may induce fibronectin synthesis against oxidative stress via PRDX 5 expression.

P622**Board Number: B873****CD47-SIRP α interactions mediate MMP expression.**J.B. Slee^{1,2}, K.A. Clarke^{2,3}, R.J. Levy^{2,3}, S.J. Stachelek^{2,3};¹Department of Pediatrics - Division of Cardiology, The Children's Hospital of Philadelphia, Philadelphia, PA, ²University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA, ³Pediatrics - Cardiology, The Children's Hospital of Philadelphia, Philadelphia, PA

Implanted medical devices and materials trigger an inflammatory reaction resulting in adsorption of blood proteins and platelets, monocyte/macrophage adhesion, and the release of pro-inflammatory cytokines, all of which contribute to clinical complications. Our lab has focused on appending recombinant CD47 to implanted polymers as a way to prevent material-induced inflammation and increase the long-term biocompatibility of medical devices. CD47 is a ubiquitously expressed transmembrane protein with a role in immune evasion and shows promise at conferring biocompatibility when appended to polymeric surfaces. SIRP α , the cognate receptor for CD47, is an immunoreceptor tyrosine-based inhibitory motif (ITIM)-containing transmembrane protein expressed on cells of myeloid origin. Recent work from our laboratory showed that the anti-inflammatory capacity of CD47-SIRP α interactions extend beyond its canonical role as an inhibitor of phagocytosis. Specifically a microarray analysis of whole blood exposed to immobilized CD47 surfaces showed a 100-fold increase in select matrix metalloproteinase (MMP) transcription. MMPs are ubiquitously expressed endopeptidases involved in degrading extracellular matrix, a process involved in the later stages of material-induced inflammation. In these studies, we test the hypothesis that CD47-SIRP α interactions increase MMP expression at the protein level. Whole human blood exposure to immobilized CD47 surfaces results in increased expression of MMP7 in plasma as determined by western blotting compared to control. The increase in MMP7 protein levels corresponded to increased enzymatic activity assessed by zymography compared to control. To validate the role of the CD47/SIRP α signaling pathway in mediating the increase in MMP7 expression and activity, THP-1 cells (monocyte-derived macrophage cells) transduced with SIRP α shRNA were used. SIRP α shRNA THP-1 cells failed to exhibit the increased MMP7 expression characteristic of exposure to polymeric tubing, establishing the necessity of SIRP α . Lastly, a rat sub-dermal implant model showed increased MMP7 staining in tissue surrounding CD47-modified implants after 7 days. Collectively, these data support the role of MMP7 activation downstream of the CD47-SIRP α interaction in preventing implanted biomaterial-induced inflammation.

P623**Board Number: B874****Proteolysis of Decellularized Extracellular Matrices Generates a Significant Amount of Fibronectin Fragments.**A. Ramanathan¹, N.W. Karuri¹;¹Chemical and Biological Engineering, Illinois Institute of Technology, Chicago, IL

Fibronectin degradation has been closely associated with a number of pathologies ranging from periodontal disease, to wound repair and cancer. Excessive inflammation in the wound is believed to result in increased fibronectin proteolysis and impaired wound healing. We studied the impact of fibronectin degradation on decellularized extracellular matrices. These were treated with alpha chymotrypsin and proteolysis stopped at different time points. The content of the protease solution as well as that of the immobilized matrices was examined by immunoblotting and Bicinchoninic Acid assays. Fibronectin was 5.0% of the total decellularized matrix but was extremely susceptible to proteolysis; after 15 minutes of protease treatment 74.5% of the fibronectin in the decellularized matrix was degraded while there was an 11.1% decrease in the total protein content in the extracellular matrix. A cell adhesion assay was used to quantify NIH3T3 mouse fibroblast adhesion, spreading and actin extensions when seeded on the decellularized extracellular matrices and on coverslips incubated with solutions of the proteolyzed extracellular matrices. Cell number, cell area and actin extensions within the extracellular matrix decreased with increased proteolysis time. Interestingly, cell adhesion and spreading positively correlated with proteolysis time in cells cultured on coverslips coated with extracellular matrix fragments. This study demonstrates that in a high protease environment, fibronectin in the extracellular matrix is more susceptible to degradation than the total extracellular matrix protein content and places weight on bioengineering strategies to stabilize it against proteolysis as a therapeutic strategy.

P624**Board Number: B875****A PROTEOLYTICALLY STABLE FIBRONECTIN THAT BINDS CELLS AND GELATIN.**N.W. Karuri¹, A. Ramanathan¹, C. Zhang¹;¹Chemical and Biological Engineering, Illinois Institute of Technology, Chicago, IL

Excessive proteolysis of fibronectin (FN) has been implicated in impaired poor wound healing, periodontal disease and tumor invasion. We previously reported two strategies for stabilizing soluble FN against proteolysis; the first conjugated polyethylene glycol (PEG) through cysteine residues and the second conjugated PEG chains of varying molecular weight on lysine residues. Cysteine PEGylation of FN increased proteolytic stability, did not perturb cell binding activity but resulted in poor gelatin binding. On the other hand, PEGylation of FN via lysine residues resulted in increased resistance to proteolysis

with increasing PEG size, but an overall decrease in biological activity, as characterized by cell and gelatin binding. Our latest method to stabilize FN against proteolysis screens the gelatin binding domain during lysine PEGylation. FN is PEGylated while bound to gelatin Sepharose beads with 2, 5 and 10 kDa PEG precursors. This results in partially PEGylated FN molecules with higher stability than native FN and whose proteolytic stability increases with PEG molecular weight. Unlike completely PEGylated FN, partially PEGylated FN has cell adhesion, gelatin binding and matrix assembly responses that are comparable to native FN. This is new evidence of how PEGylation variables can be used to stabilize FN while retaining its activity. The conjugates developed herein can be used to dissect molecular mechanisms mediated by FN stability, and address the fundamental problem of FN degradation in a number of pathologies.

P625

Board Number: B876

Tenascin-C regulates macrophage behavior during tissue repair after myocardial infarction in mouse model.

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Tenascin-C (TN-C) is a matricellular protein generally expressed at high levels during embryonic development and tissue remodeling in response to injury. It is sparsely detected in normal adults but transiently upregulated in pathological heart closely associated with tissue injury and inflammation such as acute myocardial infarction. The aim of this study was to investigate the effects of TN-C on ventricular remodeling after myocardial infarction and elucidate the molecular mechanism. Eight to 10 weeks old male wild type (WT) and TN-C knock-out (KO) mice were divided into 4 groups of WT+Sham, KO+Sham, WT+myocardial infarction (MI) and KO+MI. In WT+MI, the expression of TN-C was highly up-regulated at day 3, peaked at day 5 and then down-regulated at day 7. Fluorescence activated cell sorting (FACS) analysis showed that deletion of TN-C significantly increased that the ratio of M2 macrophage (CD45+/ F4/80+/ CD206 high) at 7 day, while the ratio of M1 macrophage (CD45+/ F4/80+/ CD206 low) were significantly decreased at day 5 compared with that of WT+MI group. RT-PCR analysis showed that the expression of IL-10, an anti-inflammatory cytokine, was significantly higher in KO+MI than WT+MI. In chronic phase, 12 weeks post-MI, although the survival rate of both WT+MI (48.3%, 14 of 29 mice) and KO +MI (55.6%, 15 of 27 mice) groups had no significant difference, TN-C KO group had the significantly better cardiac function than WT had (LVEF, 19.02±6.31% vs 10.63±4.43%; p

P626**Board Number: B877****Antagonistic effects of platelets and erythrocytes on blood clot contraction.**V. Tutwiler^{1,2}, R.I. Litvinov³, T. Lebedeva³, C. Nagaswami³, D.B. Cines³, J.W. Weisel³;¹Department of Cell and developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA, ²Drexel University, Philadelphia, PA, ³University of Pennsylvania School of Medicine, Philadelphia, PA

Blood clot contraction plays a critical role in stopping bleeding (hemostasis), wound healing, and restoring blood flow past obstructive thrombi. Contraction results in volumetric shrinkage of the clot, redistribution of platelets and fibrin polymer to the surface of the clot, and deformation of naturally biconcave erythrocytes into compressed, tightly packed, polyhedral erythrocytes (polyhedrocytes). To examine the role of blood cells in clot contraction, reconstituted blood was clotted through the addition of Ca^{2+} and thrombin. Reconstitution allowed for the platelet and/or erythrocyte levels to be varied while keeping other blood components constant. Microstructure of the clots was examined by scanning electron microscopy. Dynamic viscoelastic properties and the contractile force of the clot were determined using high precision rheology. Kinetics of contraction was quantified using an optical analyzer system, which continuously monitors changes in clot size by tracking clot-induced light scattering with time. Morphologically contracted clots revealed a packed core of polyhedrocytes surrounded by a platelet-fibrin meshwork. The mechanical nature of this cellular deformation was confirmed through the formation of polyhedrocytes when blood was centrifuged without clotting. The platelet-fibrin meshwork was able to generate the forces needed to compact the erythrocytes; the rate and degree of clot contraction depended linearly on the platelet count ($R^2=0.99$). Platelet levels $>500\text{k}/\mu\text{l}$ and $250\text{-}300\text{k}/\mu\text{l}$ resulted in more than a significant 30% and 15% increase in clot contraction, respectively, when compared to the lowest platelet concentration (40% resulted in a 30% decrease (p60% increase in the contractile forces generated by the platelet-fibrin meshwork when compared to samples without erythrocytes. These results reveal a complex effect of cellular composition on the course and degree of contraction, mechanical properties of the clot, and the contractile force. Notably, erythrocytes turned out to dampen the platelet-driven clot contraction, which may affect the formation and properties of a hemostatic plug in pathophysiological conditions with varying levels of erythrocytes, such as anemia and polycythemia.

P627**Board Number: B878****A lectin counterstain for immunofluorescence studies of equine hoof lamellar tissue and cultured keratinocytes.**R. Linardi¹, R. Clark², H. Galantino-Homer³;¹Clinical Studies, University of Pennsylvania, Kennett Square, PA, ²STEM and Health Division, Cumberland County College, Vineland, NJ, ³University of Pennsylvania, Kennett Square, PA

Equine laminitis is a common, painful, debilitating condition affecting the extensively interdigitated epidermal-dermal lamellar interface that suspends the distal phalanx bone within the hoof capsule and is a leading cause of disability in horses, often necessitating euthanasia. The objectives of this study were to develop a counterstain to facilitate histological and histopathological studies of the complex, multi-layered epidermal and dermal lamellae and to characterize this counterstain in cultured lamellar keratinocytes. A panel of seven tetramethylrhodamine isothiocyanate (RITC)-conjugated lectins (Lectin kit RLK-2200; Vector Laboratories, Inc., Burlingame, CA) was used to stain paraformaldehyde-fixed/sucrose-dehydrated lamellar tissue cryosections and paraformaldehyde-fixed cultured lamellar keratinocytes, which were then visualized by confocal microscopy. Of the seven lectins investigated, RITC-conjugated wheat germ agglutinin (WGA) displayed the most potential for utility as a counterstain. It selectively labels dermal extracellular matrix fibers and epidermal cell membranes/perimembranous regions in tissue sections of horse hoof lamellae, thereby facilitating the visualization of secondary dermal lamellae and individual epidermal lamellar keratinocytes. RITC-WGA also localizes to the cell membrane/perimembranous region of cultured keratinocytes. RITC-WGA was next applied to tissue cryosections and cultured keratinocytes to test its utility as a counterstain following indirect immunofluorescence staining for subcellular markers. As a counterstain, RITC-WGA is compatible with indirect immunofluorescence of tissue cryosections and cultured keratinocytes and augments interpretation of indirect immunofluorescence antigen localization. RITC-WGA can serve as a rapid, simple, economical counterstain for indirect immunofluorescence studies of the equine hoof and cultured keratinocytes.

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P628**Board Number: B879****The Influence of Suspended and Aligned Fibrous Environment on Mesenchymal Stem Cell Behavior.**T.L. Popielarczyk¹, A.S. Nain², J.G. Barrett³;¹Biomedical and Veterinary Sciences, Virginia-Maryland College of Veterinary Medicine, Leesburg, VA,²Department of Mechanical Engineering, College of Engineering, Virginia Tech, Blacksburg, VA,³Department of Large Animal Clinical Sciences, Virginia-Maryland College of Veterinary Medicine, Leesburg, VA

Tendon injury occurs through cumulative damage to naturally aligned extracellular matrix (ECM) causing cellular repair mechanisms to be overwhelmed. Compared to conservative management, recent advances in cell therapy have demonstrated some improvement in tendon healing. Optimization of mesenchymal stem cell (MSCs) therapy requires a better understanding of how these cells are influenced by their aligned topographical environment. We are investigating cellular response of equine bone marrow MSCs on specifically designed nanofiber scaffolds fabricated using a non-electrospinning technique (STEP). We hypothesize that equine bone marrow MSCs will differentiate into tenocytes on scaffolds that mimic native tendon ECM dimensions and alignment, through use of suspended scaffolds composed of nanofibers. MSCs were grown on 4 different designs including suspended nanofibers in parallel and intersecting orientation and supported nanofibers in parallel and suspended orientation acting as controls. Endpoints of MSC differentiation and ECM production (collagen and glycosaminoglycan) were determined through quantitative PCR and biochemical analysis respectively over 14 days. Results have shown that equine bone marrow MSCs exhibited tenocyte-like characteristics when interacting with nanofibers of suspended and parallel orientation. It was observed that cells elongated along parallel fibers in a tenocyte-like morphology, expressed upregulation of scleraxis on day 14 and expressed an upregulation of type I collagen by day 7. These cells also showed an increase production of both collagen and glycosaminoglycan content in culture media over 14 days of cell culture. These findings provide evidence of tenogenic differentiation on fibers of suspended and parallel orientation. Further modifications to the STEP platform and the study of MSCs in topographical environments may help to elucidate the parameters needed to stimulate tenogenesis.

P629**Board Number: B880****Investigating the B-LINK: an adhesion system that links neighboring basement membranes.**D.P. Keeley¹, M. Morrissey¹, D.R. Sherwood¹;¹Biology Department, Duke University, Durham, NC

Basement membranes (BM) are specialized extracellular matrices that surround most tissues in metazoans. While adhesion systems that mediate cellular attachment to basement membranes (BMs) are well known, how juxtaposed BMs associate with one another, as happens between the glomerular and endothelial BMs in the kidney and astrocyte and pericyte BMs in the blood brain barrier, is unclear. Work from our lab has recently identified a complex, termed the B-LINK, that functions to connect juxtaposed BMs together in *C. elegans* during uterine-vulval morphogenesis. The core components of this complex are integrin, plectin, and hemicentin. All these proteins are highly conserved in mammals, suggesting this may be a conserved system for attaching neighboring tissues through their adjacent BMs.

In *C. elegans*, the B-LINK connects the uterine utse cell and the epidermal seam cells by linking their juxtaposed BMs. This connection is necessary for maintaining integrity of uterine tissue during uterine muscle contractions. Perturbation of B-LINK components results in failure of the utse-seam cell attachment and expulsion of uterine and other internal tissues through the vulval opening following the start of uterine muscle contraction, this is known as the 'Ruptured vulval' phenotype (Rup). Through a GO search, we have identified 23 genes that exhibit a Rup phenotype when mutated or knocked down by RNAi. We are employing a compound microscope screen of RNAi mediated knockdown of these genes to determine if the utse-seam cell junction remains intact. If the Rup phenotype appears to be caused by separation of the utse-seam cell junction, this would indicate a potential B-LINK component. Candidates identified by this screen will then be assayed for localization of the gene product either in the utse cell or in the BMs at the utse-seam cell junction. Using this approach, we have already identified an additional B-LINK component, fibulin-1, which is also highly conserved in mammals. When knocked down by RNAi, fibulin exhibits the Rup phenotype and a translational reporter shows fibulin localization to the BM at the utse-seam cell junction. Given that fibulin assembles in a hemicentin dependent manner, we hypothesize that fibulin reinforces hemicentin, serving as an extracellular anchor for the B-LINK. Together, our work aims to identify proteins that are key regulators of the B-LINK in *C. elegans* and determine if they play conserved roles in attaching neighboring tissues in other metazoans.

Cell Death

P630

Board Number: B882

Dependence receptors and retrograde neuronal death after spinal cord injury.

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Following injury, axons in the mammalian spinal cord do not regenerate and functional recovery is further impeded by retrograde neuronal death. The retrograde cell death appears to be apoptotic, but little is known about its specific mechanisms. Recently, a new type of apoptosis-inducing mechanism, so-called “dependence receptors”, was described. Dependence receptors induce apoptosis when unoccupied by their ligands, but block apoptosis when bound by their ligands. Unlike in mammals, spinal cord injury (SCI) in the lamprey is followed by axon regeneration, but only about 50% of the severed reticulospinal (RS) axons regenerate, while the fate of unregenerated neurons is unknown. We reported previously that in animals allowed to survive 16 weeks after SCI, many of those neurons were TUNEL and activated caspase – positive, suggesting that cells were dying by apoptosis. The majority of these neurons also expressed Neogenin (axonal guidance receptor for RGM) and simultaneously, expression of RGM was downregulated near the transection site. Thus we hypothesized that after SCI, Neogenin function as “dependence receptor,” inducing delayed apoptotic neuronal death. To test this hypothesis, we downregulated Neogenin expression by in vivo delivery of antisense morpholino oligonucleotides (MOs) to RS neurons by retrograde transport at the time of spinal transection. Downregulation of Neogenin expression in lamprey RS neurons enhance their survival and prevent caspase activation. However, not all neurons where Neogenin expression was downregulated, survive after SCI. Inability of Neogenin downregulation alone to rescue all axotomized RS neurons may be explained by the fact that majority of apoptotic RS neurons co-expressed Neogenin and UNC5. Therefore, in our future experiments we will inject the combination of MOs for Neogenin and UNC5. Conclusions • Spinal cord injury lead to retrograde neuronal death of spinal-projecting brain neuron that apoptotic by nature.

- Activation of dependence receptors (UNC5 and Neogenin) might induce apoptosis of reticulospinal neurons.
- Inhibition of Neogenin expression by antisense MOs rescue some RS neurons from apoptotic death.

P631**Board Number: B883****RhoA proteolysis: A novel mechanism for RhoA regulation in cell stress and apoptosis.**M. Girouard¹, M. Pool¹, A. Fournier¹;¹Neurology and Neurosurgery, Montreal Neurological Institute, Montreal, QC

RhoA, a member of the Ras-related family of GTPases, is ubiquitously expressed in eukaryotic cells and is involved in many cellular functions, including regulation of the cytoskeleton. As such, it promotes the assembly of actin-myosin filaments known as stress fibers, limits actin depolymerization and stabilizes microtubules. RhoA regulates numerous cellular processes such as cytokinesis, adhesion and migration. RhoA is tightly regulated by a variety of mechanisms such as GTP-binding, phosphorylation and prenylation. Our laboratory has found that RhoA is processed through proteolytic cleavage to generate a 10kDa stable amino terminal fragment (NTF-RhoA), suggesting a novel regulatory mechanism for this protein. Our studies revealed that RhoA proteolysis is activity-dependent, and is regulated by both phosphorylation and prenylation. Furthermore, it is promoted following oxidative stress induced by hydrogen peroxide treatment and following initiation of apoptosis with Staurosporine. Investigation of the proteases involved in RhoA processing using specific protease inhibitors revealed that Calpain and Caspase activity result in degradation of NTF-RhoA. Introduction of NTF-RhoA into serum-starved Swiss 3T3 fibroblasts leads to mild stress fiber formation while introduction of the C-terminal fragment promotes the formation of nuclear actin rods, a hallmark of cell stress observed in some neurodegenerative diseases. Thus, our findings describe a novel mechanism of RhoA proteolysis occurring in conditions of cell stress and apoptosis. Future work will investigate RhoA proteolysis in neurons and its effects on growth cone collapse and neurite retraction. We are seeking to determine how this mechanism may impact neuronal regeneration following central nervous system injury.

P632**Board Number: B884****Ionizing radiation injury of the skin: Histologic and metabolomic evaluation in the pig as a model for testing the potential benefits of topical treatments for cutaneous radiation injury.**

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Destruction of the normal replacement capacity of the epidermis and loss of its barrier function after skin exposure to ionizing radiation is a major concern following a radiological event, such as a nuclear accident or “dirty” bomb. Current treatments are mainly palliative and do not reduce damage or promote repair. We have developed a model using the full-sized pig (*Sus scrofa*) to assess the responses of the skin to a range of β and x-ray radiation doses (18-48 Gy) over time. For β -ray radiation, a custom irradiation device was designed using strontium-90. For x-ray radiation, a conventional 300 kV orthovoltage x-ray device was modified to deliver the required, localized doses. Radiation was delivered to six, 4 cm diameter circular sites on each flank of ~30 kg female pigs (n=12 sites for 18 pigs). Dose and treatment sites were randomized on each pig, and the sites were visually graded and photographed every 3 or 4 days for up to 70 days. An equal number of pigs were euthanized on days 15, 35 and 70. Four 1-cm biopsies were collected from each of the 12 sites for histological processing and one 5-mm biopsy was frozen for metabolomic analysis. Visual grading revealed dose-related early and late erythema and moist desquamation. These observations correlated with histologic changes that included epidermal atrophy and dermal invasion by inflammatory cells, edema, and vascular pathology. Abnormal regenerative changes occurred at the higher radiation doses and longer post-irradiation intervals. Metabolomic analysis revealed dose-related increases in oxidative damage, prostaglandin-related molecules, mitochondrial breakdown, and bacterial penetration. These pathologic changes provide a greater understanding of the skin’s response to ionizing radiation and serve as a foundation from which potential treatments for this injury can be evaluated. Supported by the Biomedical Advanced Research and Development Authority, HHS, contract HHSO100201200007C.

P633**Board Number: B885****Caffeine inhibits angiogenesis via induction of endothelial cell apoptosis.**H. Li¹, L. Zhang², H. Jin³, S. Jin¹;¹Research Center, Affiliated Hospital of Yanbian University, Yanji, China, ²Department of anesthesiology, Meihekou Central Hospital, Meihekou, China, ³Department of Thoracic surgery, Yanbian University Affiliated Hospital, Yanji, China

Purpose: Numerous studies have shown that adenosine, adenosine receptor agonists, and/or adenosine transport inhibitors can stimulate angiogenesis. Recent study indicated that stimulation of adenosine receptor increased angiogenesis by down-regulating production of thrombospondin-1 (TSP-1). However, the effects of caffeine, as adenosine receptor antagonist on angiogenesis has not been studied yet. It is the aim of this study to determine the effect of caffeine on angiogenesis and to clarify the mechanism by which caffeine elicits such a response. **Methods:** To investigate the effects of caffeine on angiogenesis, chick embryonic chorioallantoic membrane (CAM) assay was performed, and data was analysed using image analysing program. Proliferation, migration and differentiation assays using human umbilical vein endothelial cells (HUVECs) were performed, to identify that caffeine affects on the specific step of angiogenesis. Apoptosis of HUVECs by caffeine was detected by FACS and DAPI staining. Expression of TSP-1, caspase-3 and Bcl-2 were examined through Western blotting. Immunofluorescence and DAPI staining were performed to identify the morphological change of HUVECs. **Results:** CAM assay showed that caffeine inhibited formation of blood vessels in dose-dependent manner. Caffeine had no effect on migration and differentiation of HUVECs. However, caffeine inhibited proliferation of HUVECs in both time- and dose-dependent manner. FACS analysis and DAPI staining showed that inhibitory effect of caffeine on the proliferation of HUVECs might be attributed to the induction of apoptosis. Caffeine increased the expression of TSP-1, which was abrogated by adenosine A2A/B agonist, but not affected by specific adenosine A2A agonist. These data indicate that caffeine regulates TSP-1 expression through blocking adenosine A2B receptor. Caffeine also increased caspase-3 expression and decreased Bcl-2 expression in HUVECs. **Conclusion:** We conclude that caffeine appears to block angiogenesis and that these effects may be mediated through increasing expression of TSP-1 via inactivation of adenosine A2B receptor.

P634**Board Number: B886****Developing a Model for Slow Hypoxic Injury and Vascular Degeneration in Amyloid Burdened Brains.**C.M. Floruta¹, F.L. Chaves¹, C.S. Medina¹, E.L. Bearer^{1,2};¹Department of Pathology, University of New Mexico, Albuquerque, NM, ²Biology, California Institute of Technology, Pasadena, CA

The breakdown of neurovascular systems may play a crucial role in the pathogenesis of Alzheimer's disease. However whether this breakdown initiates a degenerative mechanism or is the consequence of some other deleterious process remains unknown. We examined hippocampal pathology in double transgenic mice overexpressing a human mutant gene encoding the amyloid precursor protein (APP^{Swe/Ind}) using a combination of histochemistry and stereologic techniques. Expression of APP^{Swe/Ind} in these mice is driven by a tetracycline-sensitive promoter. Tetracycline transcriptional activator (tTA), the second transgene, is driven in turn by a CAM KIIa promoter that is only active in neurons. Thus this double transgenic construct allows us to control expression of APP^{Swe/Ind} with doxycycline. Utilizing this characteristic, we created three distinct experimental groups: A, display abeta plaque pathology and express APP^{Swe/Ind} at time of sacrifice; B, display abeta plaque pathology but do not express APP^{Swe/Ind} at time of sacrifice; and C, do not display abeta plaque pathology but do express APP^{Swe/Ind} at time of sacrifice. Stereologic investigation revealed decreased hippocampal volume in groups A(n=5) and B(n=5) when compared to group C(n=5) and age-matched wildtype (n=9) (p

P635**Board Number: B887****Real-time visualization of intracellular potassium dynamics in mouse macrophages during inflammasome-associated processes.**J.R. Yaron^{1,2}, L. Zhang¹, X. Kong¹, C.P. Ziegler¹, F. Su¹, S. Gangaraju¹, Y. Tian¹, H.L. Glenn¹, D.R. Meldrum¹;¹Center for Biosignatures Discovery Automation, The Biodesign Institute at Arizona State University, Tempe, AZ, ²School of Biological and Health Systems Engineering, Arizona State University, Tempe, AZ

Inflammasomes are caspase-1-activating, multiprotein platforms that mediate processing and secretion of pro-inflammatory cytokines and cell death in macrophages in response to host danger signals and pathogen invasion. It is well recognized that potassium efflux from the cell is a critical step for caspase-1 activation by inflammasomes in response to most stimuli. However, existing methods for directly investigating potassium in live cells are limited and most studies focus on bulk cell biochemical measurements or indirect indicators of pathway inhibition. Using novel intracellular potassium sensors and laser scanning confocal microscopy we demonstrate visualization of real-time potassium dynamics in live mouse macrophages. Specifically, we describe the kinetics of potassium efflux via the P₂X₇

purinergic receptor stimulated by exogenous ATP (active pathway) as well as the potassium ionophore nigericin (passive pathway). Further, we describe the mobilization of the mitochondrial potassium pool in response to exogenous ATP, suggesting a previously unobserved effect of P₂X₇ engagement beyond effecting solely cytosolic potassium efflux. These observations support a broader inflammasome-associated potassium response and demonstrate the applicability of an improved intracellular potassium sensor.

P636

Board Number: B888

Age Dependent Loss of α (E)-catenin Potentiates Cisplatin-induced Apoptosis in Renal Tubular Epithelial Cells.

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Cisplatin is a well-known anti-cancer drug used for the treatment of numerous solid tumors. A frequent side effect which limits the use of cisplatin therapy is nephrotoxicity. Previous studies in our lab have shown that aging is associated with loss of α (E)-catenin in aged kidney and aged kidney is more susceptible to cisplatin injury. We hypothesize that the loss of α (E)-catenin could increase susceptibility to cisplatin injury. To study the effects of reduced α (E)-catenin, a cell line with stable knockdown of α (E)-catenin (C2 cells) was used; NT3 is non-targeted control. C2 cells exhibited a significant loss of viability as determined by MTT assay compared with NT3 cells after cisplatin challenge, but showed no difference in lactate dehydrogenase (LDH) leakage. Increased caspase3/7/8/9 activation and PARP cleavage was observed in C2 cells after cisplatin treatment. Furthermore, blocking apoptosis using caspase 8, caspase 9, or pan-caspase inhibitors can completely abolish the difference in susceptibility between NT3 and C2 cells. The expression of α (E)-catenin was further decreased after cisplatin treatment, both in C2 and NT3 cells. Importantly, increased *in situ* apoptosis were detected in aged kidney after cisplatin challenge. In conclusion, loss of α (E)-catenin increases apoptosis of tubular epithelial cells which contributes to the increased nephrotoxicity induced by cisplatin in aged kidney.

P637

Board Number: B889

Characterization of Novel Stress Granule Components in Cancer Cells.

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The exchange of macromolecules between the cytoplasm and nucleus is essential for the viability of all eukaryotes. Nuclear transport relies on soluble factors, in particular adaptors and carriers, as represented by members of the importin- α and importin- β families. In addition to their well-established roles in nuclear transport, importin- α proteins also serve other functions. These range from

transcriptional regulation to control of proliferation in cancer cells. Some of these diverse functions are related to the stress response and in particular to the biology of stress granules (SGs). SGs are cytoplasmic compartments formed when cells are exposed to stress; they contain RNA-binding proteins and translationally arrested mRNAs. SGs play a protective role in cells, and a growing number of studies link SGs to various human diseases, such as cancer.

Here, we evaluated the response of soluble transport factors to different stressors in tumor cells. Since humans synthesize seven importin- α members that belong to three subfamilies with different cargo preferences, we focused on one representative of each group. We demonstrate that stress targets members of all three importin- α subfamilies, importin- α 1, importin- α 4, importin- α 5, as well as their binding partner importin- β 1 to SGs, but not to P-bodies. This SG association is specific and not a general property of nuclear carriers, as the nuclear exporter CAS did not concentrate in SGs. The physiological relevance of nuclear carriers in SGs is currently not understood. To address this question, we assessed their interaction with poly(A)-RNA, a major constituent of SGs. Our results establish, for the first time, that importin- α 1, but not importin- α 4 or α 5, specifically associates with poly(A)-RNA. This association occurs in a stress-depending fashion.

Taken together, we identified essential nuclear transport factors as novel SG constituents. Our work therefore sheds new light on the expanding functions of the nucleocytoplasmic transport apparatus. Moreover, it emphasizes the links between transport factors, the stress response and RNA biology. We propose that importin- α proteins, through their association with SGs, promote the survival of cancer cells under the stressful conditions they encounter in their hosts.

P638

Board Number: B890

BH3-in-groove dimer interface that initiates mitochondrial Bax oligomerization rearranges to form a pore complex with an additional helix 9 dimer interface.

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Proapoptotic Bax induces mitochondrial outer membrane permeabilization (MOMP) by forming oligomeric pores in the MOM. The assembly process and structure of the pores are largely undefined. Here, we revealed the key structural features of the pore using site-specific crosslinking and compartment-specific labeling approaches combined with mutational analysis. The BH3-in-groove dimer interface that was previously observed in crystals was indeed formed on the MOM surface by the activated integral Bax. After helix 5, a part of the groove, was released into the MOM, the remaining interface with helices 2, 3 and 4 was rearranged to another conformation. Another dimer interface was formed inside the membrane by two helices 9 that were either intersected or aligned in parallel. Large oligomers were generated by combinations of a dimer interface on the MOM with a dimer interface in

the MOM. A mutation in the groove that disrupted the BH3-in-groove interface inhibited not only the formation of other three interfaces but also the MOMP by Bax, suggesting that the formation of BH3-in-groove interface initiates a process resulting in the other three interfaces and an oligomeric pore in the MOM. In contrast, a mutation in helix 9 that disrupted the helix 9 dimer interfaces affected neither the BH3-in-groove and helices 2-3-4 dimer interfaces nor the MOMP, indicating that the helix 9 interfaces are dispensable.

P639

Board Number: B891

Gas phase extract of cigarette smoke and its cytotoxic components induce cell death via protein kinase C and NADPH oxidase.

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Cigarette smoking is a major risk factor for several diseases, such as cardiovascular diseases, chronic pulmonary obstructive diseases, and several forms of cancer. Cigarette smoke is reported to contain more than 4,000 chemical compounds. Recent studies indicate that cigarette smoke contain stable components which have the potential to stimulate cellular reactive oxygen species (ROS) production. Cigarette smoke consists of two phases: tar phase containing nicotine and gas phase. In view of human health, the gas phase is more important, because it can pass through the lung alveolar epithelium to reach the circulation, and induces cytotoxicity in tissues remote from the lung. However, the chemical compounds responsible for cytotoxicity in the gas phase of cigarette smoke and molecular mechanism of their cytotoxicity have not been elucidated. The purposes of this study are identification of cytotoxic compounds in cigarette smoke gas phase and elucidation of molecular mechanisms for their cytotoxicity. The gas phase extract of cigarette smoke (nicotine- and tar-free cigarette smoke extract, CSE) was prepared by passing the main stream of cigarette smoke through Cambridge filter to remove tar phase containing nicotine and bubbling it in phosphate-buffered saline. For determination of cytotoxicity of CSE, MTS reduction assay, lactate dehydrogenase (LDH) leakage assay, propidium iodide (PI) uptake assay, and DNA fragmentation assay were used. The CSE induced protein kinase C (PKC)- and NADPH oxidase (NOX)-dependent ROS production, cell membrane damage, and cell death. Intracellular Ca²⁺-dependent PKC activation was induced by CSE. To identify the cytotoxic components in the CSE, the CSE was fractionated into nine fractions by reverse-phase HPLC, each fraction analyzed for cell membrane damage by PI uptake assay, and two positive fractions were obtained. Using LC/MS and GC/MS in combination with functional assays, the unsaturated carbonyl compounds such as acrolein (ACR), methyl vinyl ketone (MVK) and 2-cyclopentene-1-one (CPO) were identified as the major compounds responsible for the cytotoxic activity in these two fractions of the CSE. Incubation for a short period of time (4 h) with ACR and MVK but not CPO induced both cell membrane damage (determined by PI uptake and LDH leakage assays) and cell death (determined by MTS reduction and DNA fragmentation assays) in rat C6 glioma cells in a PKC- and NOX-dependent manner. In contrast,

CPO induced cell death alone without cell membrane damage in a manner independent of PKC and NOX only after incubation for a longer period of time (24 h). These results show that ACR and MVK are responsible for acute cytotoxicity of the CSE through PKC- and NOX-dependent mechanism, whereas CPO is responsible for delayed cytotoxicity of the CSE through PKC- and NOX-independent mechanism.

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Board Number: B892

Cytoplasmic translocation of DGKzeta exerts a protective effect against p53-mediated cytotoxicity.

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The transcription factor p53 plays a crucial role in various stress responses, such as DNA-damage, hypoxia, hyperproliferation, and oncogene activation. In response to cellular stresses, p53 triggers various cellular reactions that engender DNA repair, cell cycle arrest, apoptosis, senescence, differentiation, and inhibition of angiogenesis. Therefore, p53 protein levels and activity need to be kept under tight control. Diacylglycerol kinase (DGK) consists of a family of isozymes that metabolize a lipid second messenger diacylglycerol. Of the family, DGKzeta, containing a nuclear localization signal (NLS), localizes primarily to the nucleus in various cell types and is involved in a variety of pathophysiological cellular responses through the metabolism of DG. Recently, we have reported that excitotoxic stress induces DGKzeta nucleocytoplasmic translocation in hippocampal neurons. Although p53 activation is implicated in the apoptotic cell death under stress conditions, it remains unclear how DGKzeta nucleocytoplasmic translocation is involved in p53-mediated stress response mechanisms. In this study, we examined a pathophysiological link between DGKzeta cytoplasmic translocation and p53-mediated cytotoxicity after doxorubicin (DOX)-induced DNA damage. For this purpose, using cellular and organismal models, we investigated the effect exerted by DGKzeta on p53, i.e., how increased cytoplasmic pool of DGKzeta and attenuated nuclear DGKzeta affect p53 function after apoptotic stimuli. We show here that cytoplasmic DGKzeta attenuates p53-mediated cytotoxicity against DOX-induced DNA damage by facilitating cytoplasmic anchoring and degradation of p53 through a ubiquitin-proteasome system. Concomitantly, decreased levels of nuclear DGKzeta engender down-regulation of p53 transcriptional activity. Consistent with these in vitro cellular experiments, DGKzeta-deficient brain exhibits high levels of p53 protein after kainate-induced seizures and even under normal conditions. These findings suggest that 1) DGKzeta serves as a sentinel to control p53 function under normal and stress conditions and 2) its cytoplasmic translocation is a protective stress response and attenuates p53-mediated cytotoxicity under stress conditions. This study provides a novel molecular basis for the regulation of p53-mediated apoptotic pathway.

P641**Board Number: B893****Med13p anchors cyclin C in the nucleus to prevent stress-independent mitochondrial fragmentation and stress hypersensitivity.**K. Cooper¹;¹RowanSOM, Stratford, NJ

The budding yeast cyclin C has two roles in the cell. First, in combination with Med12p, Med13p and Cdk8p, cyclin C forms a subcomplex (Cdk8 module) of the mediator that represses transcription initiation of several gene types including stress responsive loci. In its second role, cyclin C translocates to the cytoplasm in response to oxidative stress. In the cytoplasm, cyclin C associates with the mitochondria to promote extensive fission and programmed cell death. Therefore, releasing cyclin C from the nucleus represents a critical decision point in the cellular response to stress [1]. We demonstrate that Med13p represents the nuclear anchor that retains cyclin C in the nucleus in unstressed cells. Deleting MED13 allows cyclin C relocation to the cytoplasm in the absence of stress. This event has three important consequences for the cell. First, the mitochondria undergo constitutive extensive fission. Second, since this fragmentation prevents mitochondrial DNA repair, large deletions occur in the mitochondrial genome resulting in loss of organelle function. Finally, mitochondrial hyper-fission hypersensitizes cells to programmed cell death [2]. Further studies revealed two independent mechanisms that dissolve the Med13p-cyclin C interaction. First, cyclin C is phosphorylated by the Slr2p MAP kinase [3]. This modification causes partial release of cyclin C from Med13p. However, final dissolution of the interaction requires Med13p proteolysis. Med13p destruction requires Cdk8p kinase activity and the 26S proteasome. Taken together, this study defines a complex molecular switch controlling cyclin C subcellular localization and ultimately mitochondrial shape, function and sensitivity to cytotoxic agents. This study reveals a new role for Med13p in maintaining mitochondrial maintenance and preventing aberrant activation of the programmed cell death pathway by restricting cyclin C to the nucleus in non-stressed cells.

1. Cooper KF, Khakhina S, Kim SK, Strich R (2014). Stress-induced nuclear-to-cytoplasmic translocation of cyclin C promotes mitochondrial fission in yeast. *Dev Cell* 28(2): 161-173. doi: 10.1016/j.devcel.2013.12.009.
2. Khakhina S, Cooper KF, Strich R (2014). Med13p prevents mitochondrial fission and programmed cell death in yeast through nuclear retention of cyclin C. *Mol Biol Cell*. doi: 10.1091/mbc.E14-05-0953.
3. Jin C, Strich R, Cooper KF (2014). Slr2p phosphorylation is required for the stress-induced cytoplasmic translocation and destruction of the yeast transcriptional repressor cyclin C. *Mol Biol Cell* 25(1396-1407). doi: 10.1091/mbc.E13-09-0550.

P642**Board Number: B894****Complexes of platelet factor 4 with pathogenic anti-platelet factor 4/heparin antibodies induce platelet activation, apoptosis, and release of microparticles.**T.A. Nevzorova¹, E.R. Mordakhanova¹, I.A. Andrianova¹, L. Rauova^{2,3}, R.I. Litvinov²;¹Department of Biochemistry, Kazan Federal University, Kazan, Russia, ²University of Pennsylvania School of Medicine, Philadelphia, PA, ³Children's Hospital of Philadelphia, Philadelphia, PA

Platelets are blood cells that have many important functions, including their well-known role in stopping bleeding (hemostasis) and thrombosis. Platelet functionality is affected in (patho)physiological conditions by a number of bioactive blood components, such as a self-secreted chemokine platelet factor 4 (PF4) and its complexes with heparin and anti-PF4/heparin antibodies. A subset of these antibodies cause heparin-induced thrombocytopenia (HIT), a severe immunological complication of heparin therapy, in which platelets aggregate and cause thrombotic obstruction of the microvasculature; however, the mechanisms and functional cellular consequences of such platelet activation remain largely unclear. To get insight into the link between autoimmunity and platelets in HIT, we incubated resting platelets in vitro with either purified recombinant human PF4 (10 µg/ml) or isolated pathogenic monoclonal mouse anti-human-PF4/heparin antibodies (named KKO) (50 µg/ml) or the immune complexes between PF4 and KKO formed by mixing them at the same final concentrations. Gel-filtered platelets isolated from the blood of healthy donors were treated during 15-60 min followed by flow cytometry using apoptotic markers Annexin V-FITC and MitoTracker Red CMXRos. Annexin V binding characterize the exposure of the procoagulant phosphatidylserine on the outer platelet membrane during activation and apoptosis, while lowering of intensity of the MitoTracker reflects a decrease of the mitochondrial membrane potential during early stages of apoptosis. We revealed that in the presence of the KKO/PF4 complexes the number of Annexin V-FITC-positive signals increased with a concurrent shift of the particle size towards smaller structures, likely representing formation of platelet-derived microparticles. Staining of platelets with the MitoTracker revealed that the KKO/PF4 complexes significantly reduced the mitochondrial membrane potential, which is indicative of platelet apoptosis. Compared to the KKO/PF4 complexes, PF4 by itself induced qualitatively similar but substantially less pronounced changes in the platelet status. KKO alone had no detectible effects on the platelets. The results suggest that immune complexes of the pathogenic anti-PF4/heparin antibodies with PF4 induce platelet activation and apoptosis accompanied by release of platelet-derived procoagulant microparticles, which may contribute to the thrombotic component of HIT. In addition, the data suggest that PF4 released at the sites of inflammation and injury by activated platelets promotes their programmed death and maybe subsequent scavenging. (Research supported by the Program of Competitive Growth of Kazan Federal University)

P643**Board Number: B895****Estrogen improves osteocyte viability by inhibiting apoptosis and enhancing autophagy in alveolar process of ovariectomized rats.**R.F. Silva¹, G.R. Sasso¹, E. Sasso-Cerri², M.J. Simões¹, P.S. Cerri²;¹Morphology and Genetics, Federal University of São Paulo (UNIFESP), São Paulo, Brazil, ²Morphology, Univ Estadual Paulista - UNESP, Araraquara, Brazil

Osteocyte viability is influenced by several factors, such as estrogen status and ageing. Autophagy, a cell survival mechanism, has also been shown to maintain osteocyte viability by preventing accumulation of cellular stress. However, it is not known whether estrogen improves osteocyte survival by promotion of autophagy. Thus, we aimed to investigate whether estrogen influences osteocyte survival by regulating autophagy. Sixty-six adult female rats were sham-operated (SHAMG) or ovariectomized (OVXG). After 21 days, the rats received daily subcutaneous injection of 30µg/Kg of body weight of estrogen (OVXEG) or vehicle solution (OVXG) for 15, 30 and 45 days. Twelve non-estrogen-treated SHAM and OVX female rats were used as baseline groups. Afterwards, the rats were euthanized and the maxillae containing the alveolar processes of the first molars were fixed and embedded in paraffin. Sagittal sections were subjected to immunohistochemistry for detection of apoptosis (cleaved caspase-3, bcl2 and TUNEL method) and autophagy (beclin1, LC3II and p62). Some fragments of maxilla were also fixed and embedded in araldite for transmission electron microscopy. Significant increase in the incidence of caspase-3, TUNEL and p62-positive osteocytes was observed in the OVX baseline group in comparison to SHAM baseline. On the other hand, the frequency of beclin-1 and LC3II-positive osteocytes was significantly lower in the OVX baseline group. In all periods, a significant increase of TUNEL-positive osteocytes was observed in the OVXG in comparison to OVXEG. In addition, high incidence of caspase-3-immunolabeled osteocytes was also found in the OVXG at 15 and 45 days. In these periods, a significant reduction in the incidence of beclin1-positive osteocytes was found in the OVXG in comparison to OVXEG. At 45 days, a significant increase in the incidence of p62-positive osteocytes and significant reduction in the incidence of LC3II was observed in the OVXG in comparison to OVXE. Significant differences were not verified in the frequency of bcl2-immunolabeled osteocytes among the groups. Ultrastructural analysis revealed osteocytes containing peripheral condensed chromatin; some abnormal osteocytes exhibiting cytoplasmic protrusions were also found. Occasionally, some bone lacunae contained apoptotic bodies derived from osteocytes. Our findings point to a possible correlation between autophagy reduction and apoptosis increase in osteocytes, under estrogen deficient condition. On the other hand, estrogen replacement improved osteocyte survival by stimulating autophagy and inhibiting apoptosis in the alveolar process of ovariectomized rats. These findings indicate that autophagy plays an important role in osteocyte survival.

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P644**Board Number: B896****Neutrophils of Patients with Chagas Positive Serology in Autologous Culture.**F.M. Rodriguez¹, A.D. Orquera¹, M.R. Maturano¹, N.S. Infante¹, I.T. Novak^{1,2};¹Institute of Cell Biology, Faculty of Medical Sciences, National University of Cordoba, Cordoba, Argentina, ²Institute of Cell Biology, Natl Univ Cordoba Fac Med, Cordoba, Argentina

Neutrophils or polymorphonuclear (PMN) leukocytes play a crucial role in protecting against infections and recent evidence suggests that they are involved in a key role in effector and regulatory mechanisms of the innate and adaptive responses (Mantovani et al., 2011; Phillipson and Kubes, 2011). Currently there is controversy about the half life of these cells (Kolaczowska and Kubes, 2013). In inflammatory conditions, PMN can release neutrophil extracellular traps (NETs) (Brinkmann et al, 2004, Kolaczowska and Kubes, 2013) composed of chromatin, histones and granule proteins, which sometimes lead to NETosis. Objectives: perform an ultrastructural study to observe the morphology of PMN in relation to characteristics of viability, apoptosis, or NETosis, at different times of autologous culture in healthy people and with positive serology for Chagas who donate blood to the blood bank of the UNC. From healthy human blood samples (n = 10) and with positive Chagas serology (n = 6), anticoagulated with heparin (donated by the blood bank, UNC, in anonymity, with serology data) autologous leukocyte cultures were performed in TC199 medium (SIGMA, St. Louis, MO). Samples of cell culture to 1, 3, 20, 24 and 48 h were subjected to stains standard, H/E, ATO, Giemsa microscopy and processing techniques for ultrastructural study with an electron microscope MET: Zeiss LEO-neoi. Results: in culture samples with positive serology for Chagas PMN were observed ultrastructural features of viability at all times even at 48h; in one case it was observed an NETosis-compatible image. In healthy people, after 3 h of culture were observed images of apoptosis. Most of PMN after 20 h showed apoptotic characteristics. We open questions for further study: what stimuli contribute to increase the half-life of PMN in Chagas? which stimuli induce to NETosis?, is this NETosis "vital" or "suicide"?

P645**Board Number: B897****Effect of Regular and Light Cigarette Smoke Extract on Human Lung Epithelial Cells.**A. Madabushi¹, A.R. Gbemafu¹, L.Y. Kougang¹;¹Baltimore City Community College, Baltimore, MD

Cigarette smoking continues to be a leading preventable cause of morbidity and mortality worldwide. Cigarette smoke mediates oxidative as well as inflammatory events directly effecting airway and alveolar linings. This eventually leads to smoking related pulmonary diseases and disorders. One such chronic disorder of unknown etiology is idiopathic pulmonary fibrosis (IPF). The complete disease biology of IPF is an area of active research; however one of the key determinant of initiation and progression of IPF is believed to be based on the response of alveolar epithelial cells type II (AEC II) to the wound healing

process post injury. The purpose of this study is to acquire a better understanding of how short term exposure of cigarette smoke from regular or light cigarette impacts AEC II and contributes to the initiation of IPF. Out of the two popular brands tested in the study, morphology studies and cell viability assays in AEC II cells have shown similar cell death upon treatment with smoke extract from light or regular cigarettes for the brands tested. The wound healing assays have also shown that wound healing is substantially reduced, if the cells are treated with cigarette smoke extracted media regardless of the type (regular or light) and brand used. This indicated loss of function and viability on AEC II post cigarette smoke treatment holds the key to unravelling the initiation of IPF and similar pulmonary disorders.

P646

Board Number: B898

Investigation of intrinsic cell death using different inducers reveals differences in cell death pathways upon lysosomal leakage.

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Cell death is necessary to maintain homeostasis; however, under several conditions this homeostasis is disturbed resulting in adverse physiological and pathological outcomes. Apoptosis, the most commonly studied form of cell death can be divided in to intrinsic and extrinsic pathways. The intrinsic pathway can be activated by lysosomal leakage. Various compounds can be designed to specifically target lysosomes to activate this death stimulus, which can be used for therapeutic purposes. We were therefore interested in understanding the underlying molecular machinery leading up to cell death post lysosomal leakage.

Three different compounds were chosen to stimulate lysosomal leakage: silica particles that cause lung fibrosis and the disease silicosis; staurosporine (STS), a protein kinase inhibitor; and Leu-Leu-O-Me (LLOMe), a lysosmotropic agent. Alveolar macrophages were chosen as a cell line of choice due to their relevance to the development of silicosis. According to literature, lysosomal leakage can activate protein Bid that can further activate cytoplasmic Bax resulting in its relocalization to mitochondria and formation of pores in the outer mitochondrial membrane resulting in mitochondrial outer membrane permeabilization (MOMP). This leads to release of Smac and Cytochrome-c causing activation of Caspase-9 and -3. Activation of the latter is a point-of-no return in apoptosis. MOMP results in disruption of mitochondrial function by causing mitochondrial membrane depolarization.

Spectrally distinct genetically encoded fluorescent probes for Bid, Bax, Smac and caspase were expressed in macrophages individually or in combination. Both Bid and caspase were Förster Resonance Energy Transfer (FRET) based probes. Multiple compounds and parameters were tested utilizing a semi-throughput imaging scheme. With all three compounds, lysosomal leakage occurred within one hour and continued over time. Bax activation preceded mitochondrial membrane depolarization for both silica

and STS treatment where as for LLOMe treatment Bax activation follow mitochondrial membrane depolarization. Surprisingly for STS treated cells, Smac release from mitochondria was observed a minute prior to Bax localizing into mitochondria rather than later. FRET based Bid activation was observed post caspase activation rather than following lysosomal leakage for both silica and STS treatment. Our data therefore does not support the role of Bid in Bax activation but rather shows Bax activation to occur by yet an unknown trigger. Caspase activation and cell blebbing, a morphological characteristic of apoptosis was shown to occur simultaneously with mitochondrial depolarization in staurosporine treated cells or with mitochondrial hyperpolarization in silica treated cells.

P647

Board Number: B899

The effect of aflatoxin B1 on Jurkat T-cell growth, viability, and activation.

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Aflatoxins develop from two common mold species, *Aspergillus flavus* and *Aspergillus parasiticus*, known for the contamination of animal feeds as well as staple foods such as groundnuts, corn, rice, spices and seeds. This contamination is common in animal feed as well as food supplies in developing countries, where food-production technology is not developed enough to avoid the conditions conducive to mold growth. The metabolite aflatoxin B1 (AFB1) has been identified as a carcinogen known to cause liver cancer. Animal studies have suggested that AFB1 may also have a negative impact on immune function and clinical studies on humans exposed to aflatoxin have also shown immunosuppression. However, the direct effects of exposure to aflatoxin on human immune cells and the mechanism behind immunosuppression have not been thoroughly studied. This study investigated the effect of AFB1 on T lymphocyte activation, growth and viability. After treatment of the Jurkat T-cell line with 10 μ M AFB1 for 48 hours, microscopic viability counts and flow cytometric analysis showed that cell concentrations decreased by 50%. A 7-day treatment with 10 μ M AFB1 showed a decrease in cell concentration to 42% of the control. In order to investigate the decrease in cell concentration, Propidium Iodide (PI) and BrdU staining and flow cytometric analysis were used in order to identify alteration of the cell cycle. In Jurkat cells treated for 24 hours with AFB1, no change in the cell cycle was observed. Western Blot analysis of extracts obtained from cells pre-treated with or without AFB1 followed by stimulation with phorbol myristate acetate and phytohemagglutinin (PMA/PHA) showed the ERK MAP Kinase activation pathway was not significantly modulated by AFB1, thus suggesting that changes in cell activation were not responsible for immunosuppression. Additionally, the potential induction of apoptosis was studied using Annexin-V/PI staining and flow cytometric analysis. After a 48 hour treatment with 10 μ M AFB1 the t-cell line showed a significant increase in Annexin V+/PI- cells (early and late apoptotic cells) of approximately 50% (from an average 8.14% to 12.5%) from the untreated control. After a 7 day treatment with the same conditions, the proportion of apoptotic cells increased by 83%. These results suggest AFB1 might promote immunosuppression by significantly reducing T-cell concentrations, at least in part, by inducing apoptosis.

P648**Board Number: B900****The study of BAX oligomerization kinetics using live-cell imaging.**M. Maes¹, C. Schlamp¹, R. Nickells¹;¹Ophthalmology and Visual Sciences, Univ Wisconsin-Madison, Madison, WI

BAX, a pro-apoptotic *BCL2* gene family member, regulates the committed step of the intrinsic apoptotic pathway. Once activated, BAX forms oligomers at the mitochondrial outer membrane, which facilitate the release of cytochrome c. To date, the mechanism of BAX activation has been a primary focus of study, while the mechanism of BAX oligomerization has been secondary, and predominantly described using biochemical methodology. We propose that by studying the kinetics of BAX oligomerization using live-cell imaging, we can provide a better understanding of the underlying mechanisms of BAX oligomerization and function.

To monitor BAX oligomerization kinetics *in vitro*, HCT116^{BAX^{-/-}/BAK^{-/-}}, D407 and neuronally differentiated 661W cells transiently expressing fluorescently labeled wildtype BAX or non-oligomerizing BAX mutant protein were challenged with apoptotic stimuli, staurosporine or HDAC3 overexpression. Cells were imaged every minute for 60-75 minutes using spinning disc confocal microscopy and changes in BAX fluorescence at individual mitochondria were quantified using IMARIS 7.7. The curve fitting function of the SciPy library was used to fit the resulting quantitative data. Monitoring BAX non-oligomerizing mutants and cytochrome c release validated BAX oligomer formation in the system.

BAX oligomerization kinetics can be quantified at individual mitochondria. Oligomerization follows a sigmoidal growth function from which, we can calculate metrics for the initiation and rate of BAX oligomerization. Mitochondria in individual cells had nearly identical initiation times but exhibited varying rates of BAX oligomerization. The average rate of BAX oligomerization within a population of over 300 mitochondria from D407 cells is 0.38 ± 0.30 RFU/min and this oligomer formation, defined as the time of initiation to maximum saturation, occurs within 10-12 minutes. The distribution of BAX oligomerization rates between two divergent cell types, D407 and neuronally differentiated 661W cells, were not statistically different even when the average initiation time for each cell type varied by several hours. Finally, we observed that cytochrome c release occurred at the initiation time of oligomer formation. These preliminary results demonstrate that the study of BAX oligomerization kinetics can be a powerful tool for providing insight into the mechanism of BAX function in apoptosis.

P649**Board Number: B901****Nanoparticle-induced stress signaling and apoptosis in diverse cell lines.**A. Calixto¹, B.N. Manes¹, S.K. Alvarado¹, J.L. Brewster¹;¹Natural Science Division, Pepperdine Univ, Malibu, CA

Carbon black (CB) is a type of nanoparticle commonly found in air pollution generated through fossil fuel combustion. Exposure of cells to CB and other types of nanoparticles results in endocytosis and particle accumulation in the cytosol. In this work, we have begun to assess cellular toxicity and stress signaling pathways in cells exposed to CB. Doses of exposure ranged from 5-100 ug/ml. Size analysis of CB nanoparticles generated by sonication were shown to range upwards from 75 nm in diameter, with an average size of ~130 nm in diameter. Analysis of cell death activation was performed in several cell types, including adenocarcinomic human alveolar basal epithelial (A549), hamster fibroblasts (BHK21), and human bronchial epithelial cells (BHE16). Exposing cells over a time course of five days, CB exposure was shown to dramatically induce condensed nuclei in each cell type in a dose-dependent manner. Consistent with this finding, cell survival assays revealed CB exposure in doses of 40 ug/ml or higher to reduce cell survival in both BHK21 and BHE16 cells. Interestingly, the human A549 cells displayed only a mild sensitivity to CB exposure at high doses. Results of the cell death assay were confirmed to be apoptotic using standard nick end-labeling assay (TUNEL). CB-exposed cells displayed a transient elevation of reactive oxygen species (ROS) levels, consistent with other reports noting nanoparticle-induced stress signaling. Efforts are underway to evaluate which stress signaling pathways are responsible for triggering apoptotic death in these cells, with focused attention being applied to the endoplasmic reticulum.

Chaperones, Protein Folding, and Quality Control 1**P650****Board Number: B902****Quality control of GPI-anchored proteins in the secretory pathway.**P. Satpute-Krishnan¹, J. Lippincott-Schwartz^{2,3};¹Cell Biology and Metabolism Program, Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH, Bethesda, MD, ²Physiology Course at Marine Biological Laboratory, Woods Hole, MA, ³Cell Biology and Metabolism Program, NICHD, NIH, Bethesda, MD

Accumulation and aggregation of misfolded secretory and membrane proteins can lead to various degenerative diseases. To avoid this, cells employ quality control systems that route misfolded proteins for degradation, including the well studied ER associated degradation (ERAD) pathways. ERAD involves retrotranslocation of the protein from the ER to the cytosol for proteasomal degradation. However, not all misfolded secretory pathway proteins are efficient ERAD substrates, requiring alternate degradation

pathways. In particular, we found that misfolded GPI-anchored proteins (GPI-APs) are poorly recognized by ERAD systems. GPI-APs encompass a major class of proteins that play essential roles in critical life processes including embryogenesis, fertilization, neurogenesis, and immunity (1). The GPI-anchor is a post-translational modification conserved in all eukaryotes (1). Despite the prevalence and ubiquity of GPI-APs, little is known about how their quality control is regulated.

To elucidate how cells handle misfolded GPI-APs, we created a diverse panel of fluorescent protein-tagged misfolded variants of GPI-APs and monitored them by live cell microscopy combined with biochemical analysis. We identified a novel degradation pathway that operates constitutively but is markedly enhanced during ER stress (2). At the onset of stress, misfolded GPI-APs dissociate from ER chaperones and through requisite interactions with p24 ER-export factors leave the ER to the secretory pathway. Inhibiting this pathway through depletion of its key ER-export factor results in their aggregation in the ER. We named this stress-inducible mechanism RESET (for rapid ER stress-induced export) as it helps to reset ER homeostasis during the critical period before induction of the unfolded protein response.

Intriguingly, after release from the ER into the secretory pathway, misfolded GPI-APs transiently access the cell-surface before destruction in lysosomes, implicating plasma membrane-level quality control. Although misfolded transmembrane proteins have been shown to be downregulated from the plasma membrane after ubiquitination on their cytosolic regions, GPI-APs lack a cytosolic domain, implicating a distinct pathway for rapid downregulation of misfolded GPI-APs. We are now using the tractable experimental systems that we have developed to reveal novel mechanisms in plasma membrane quality control.

(1) Fujita, M., and Kinoshita, T. (2012) GPI-anchor remodeling: potential functions of GPI-anchors in intracellular trafficking and membrane dynamics. *Biochim Biophys Acta* 1821, p1050-1058 (2) Satpute-Krishnan et al. (2014) ER stress-induced clearance of misfolded GPI-anchored proteins via the secretory pathway, *Cell*, In Press

P651

Board Number: B903

Lipid Disequilibrium Destabilized a Subset of Membrane Proteins.

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Hundreds of distinct lipids, of varying concentrations, assemble to form biological membranes. The most abundant, phospholipids, varies according to head group structures, acyl chain length and double bounds. In eukaryotes, lipid compositions can differ widely among organelles. In most cases, the

biological significance of these differences remains unclear. However, links between disease states and lipid disequilibrium have been proposed. It was reported by several groups that change in the ratio of the most two abundant phospholipids, phosphatidylcholine / phosphatidylethanolamine (PC/PE), might cause non-alcoholic fatty liver disease (NAFLD). Recently, we reported on how budding yeast cells respond to and cope with PC/PE disequilibrium. Using lipidomic, genomic, and proteomic technologies, the data revealed that the adaptive cells responded by remodeling the protein homeostasis network without restoring lipid composition. We termed this process the membrane stress response (MSR). Interestingly, we observed that some transmembrane proteins (TP) were strongly up- and down-regulated at the genomic and proteomic levels, respectively. Diverse candidate proteins were analyzed from normal and mutant cells to characterize their stability. Results suggest the stability of certain mature TPs is affected from lipid imbalance. Furthermore, premature degradation of the candidates directly affects ER functions such as protein translocation and degradation if not for the intervention of the unfolded protein response (UPR). We are currently exploring how lipid imbalance affects the stability of such TPs.

P652

Board Number: B904

Disulfide bond formation regulates PMEL amyloid potential.

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The deposition and accumulation of amyloid protein is a common feature of many neurodegenerative diseases. However, not all amyloid proteins are associated with disease. For example, premelanosome protein (PMEL) is a functional amyloid protein that forms nontoxic amyloid fibrils that serve to template the polymerization of melanins within the melanosomes of pigment cells. PMEL is synthesized as a type I transmembrane protein and navigates the secretory pathway in a non-amyloid form; only within stage I melanosomes do proteolytically processed PMEL fragments form benign amyloid fibrils under normal physiological conditions. Here we provide evidence that disulfide bond formation with a partner protein might regulate the timing of PMEL amyloid formation, potentially preventing the formation of toxic amyloid isoforms. Results from non-reducing SDS-PAGE, size exclusion chromatography, and sedimentation velocity analyses support the existence of two covalent PMEL complexes of approximately 250kDa (complex 1) and 160kDa (complex 2). By metabolic pulse-chase and immunoprecipitation, complex 1 forms concomitant with or soon after ER exit, whereas complex 2 is generated in the late Golgi or a post-Golgi compartment. By 2-D non-reducing/reducing PAGE of metabolically labeled and immunoprecipitated material, these complexes consist of both full-length, Golgi-matured PMEL and post-Golgi cleaved PMEL fragments. No additional proteins were detected by 2-D PAGE or by mass spectrophotometric analysis of tryptic peptides from purified complex 2. Formation of both complexes is impaired by mutagenesis of cysteine 301 (C301S) within the

amyloidogenic M α domain, whereas formation of complex 2 is uniquely disrupted by mutagenesis of cysteine residues within a cysteine-rich Kringle-like domain that lies outside of the amyloidogenic region. Importantly, these mutations have very different effects on amyloid fibril formation upon transient overexpression in a non-pigment cell type, HeLa. Due to the absence of melanosomes in this system, amyloid fibrils composed of wild-type PMEL form in late endosomal multivesicular compartments. Compared to wild type PMEL, the C301S variant displays relatively normal levels of amyloid fibril formation in these compartments, whereas a C566S variant (lacking a Kringle-like domain cysteine residue) displays drastically reduced levels of amyloid fibril formation. Together, these results indicate that (1) disulfide bonds within the Kringle-like domain are necessary to chaperone the PMEL amyloidogenic domain for effective amyloid formation, and (2) complex 1 might serve to inhibit premature fibril formation, such that resolution of this species to complex 2 is necessary for normal fibril formation.

P653

Board Number: B905

Conformationally promiscuous prion-like domains coordinate RNP granule condensation/dissolution dynamics during stress.

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The formation of large supramolecular assemblies, such as stress-inducible RNP granules, is a poorly understood process. Here, we perform an extensive analysis of P bodies and stress granules in budding yeast and find that they have very distinct material properties: whereas P bodies behave as liquid droplets, stress granules adopt a solid material state, which originates from co-aggregation with misfolded proteins. Maintenance of the liquid P body state depends on the continuous action of protein disaggregases, and interference with this process results in the entrapment of P body components in stress granules. We further demonstrate that RNP assembly can be nucleated through multiple pathways and requires scaffolding factors such as misfolded proteins or RNAs. Low complexity domains can also drive RNP granule assembly, and we find that these domains do not undergo prion-like conformational conversions but function as versatile molecular adaptors that promiscuously interact with other low complexity domains or misfolded proteins. In summary, our findings reveal a high degree of adaptability and redundancy in RNP granule biogenesis, which is regulated and maintained by ATP-driven disaggregating machines.

P654**Board Number: B906****Intracellular Calcium Regulates Nonsense-Mediated mRNA Decay in Human Cells.**A. Nickless¹, E. Jackson², J. Marasa², P. Nugent¹, R.W. Mercer¹, D. Piwnica-Worms³, Z. You¹;¹Department of Cell Biology and Physiology, Washington University School of Medicine, Saint Louis, MO,²BRIGHT Institute, Molecular Imaging Center, Mallinckrodt Institute of Radiology, Washington UniversitySchool of Medicine, Saint Louis, MO, ³Department of Cancer Systems Imaging, The University of Texas

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The nonsense-mediated mRNA decay (NMD) pathway functions as an RNA surveillance system that selectively eliminates aberrant transcripts containing premature translation termination codons (PTCs). In addition, NMD acts as a gene expression regulatory mechanism that controls the levels of a number of physiological mRNAs. NMD modulates the clinical outcome of a variety of human diseases, including cancer and many genetic disorders, and may represent an important target for therapeutic intervention. To investigate the NMD pathway and to begin to develop NMD-targeting therapeutics, here we have developed a novel multicolored, bioluminescence-based reporter system that can specifically and effectively assay NMD in live human cells. Using this reporter system, we conducted a robust high-throughput small-molecule screen in human cells and, unexpectedly, identified a group of cardiac glycosides including ouabain, digoxin, digitoxin, proscillaridin and lanatoside C as potent inhibitors of NMD. Cardiac glycoside-mediated effects on NMD are dependent on binding and inhibiting the Na⁺/K⁺-ATPase on the plasma membrane and subsequent elevation of intracellular calcium levels. Induction of calcium release from endoplasmic reticulum also leads to inhibition of NMD. Thus, this study reveals intracellular calcium as a key regulator of NMD and has important implications for exploiting NMD in the treatment of disease.

P655**Board Number: B907****A mechanism of polarized inheritance of proteome damage during yeast asymmetric cell division.**C. Zhou¹, R. Li², B.D. Slaughter², J. Unruh²;¹Rong Li lab, Stowers Institute for Medical Research, Kansas City, MO, ²Stowers Institute for Medical

Research, Kansas City, MO

Formation and propagating of protein aggregates cause many aging-related and amyloid diseases. In yeast, the process of replicative aging is naturally associated with accumulation of damaged protein aggregates that are distributed asymmetrically during cell division. In a crowded cytosolic environment with many organelles, aggregate formation can be a consequence of non-native protein structures being introduced into these well-organized intracellular compartments. How do membrane-based organelles contribute to and coordinate the formation and distribution of protein aggregates is not well understood. In this study, we investigated the role of organelles in the formation, dissolution and

distribution of protein aggregates. We obtained evidence that cytosolic unfolded proteins do not aggregate spontaneously; instead, active translation and nascent polypeptides are required to initiate protein aggregation on the surface of ER, an organelle harboring a majority of translation sites. In addition to ER, mitochondria play important roles in the dynamics and asymmetric segregation of aggregates during mitosis.

P656

Board Number: B908

General occurrence of pausing in translation of the *E. coli* proteome members as studied by direct detection of nascent polypeptides.

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Detailed knowledge only recently started to accumulate about the progression of translation elongation, which is not simply a latent step in the central dogma and takes tens of seconds in prokaryotes and a few minutes in eukaryotes. Some nascent polypeptides interact with the ribosomal exit tunnel to modulate elongation speed as a means of gene regulation (1). Translational modulation could also affect the fates of the polypeptide products including folding, modification and subcellular targeting. Although the methodology of ribosome profiling has recently been used to detect events of translation pausing, we still need more direct detection of translation intermediates. We use the chemical trait of having a covalently attached tRNA moiety to detect and profile the global complement of nascent polypeptide chains or "nascentomes" in the cell (2), as well as to collect elongation profiles of individual proteome members. Thus, we examined the generality of the occurrence of translational pausing by directly detecting nascent chain intermediates in the biosynthesis of *E. coli* proteins. Combined approaches of in vivo pulse-chase labeling and cell-free translation revealed that the majority of the ~1,000 proteins examined underwent one or multiple event(s) of pausing either in vitro, in vivo, or both. Classification of the pausing events showed that some interesting correlation exists between the modes of pausing and subcellular localizations of proteins. (1) Ito, K. and Chiba, S. (2013) Arrest peptides: cis-acting modulators of translation. *Annu. Rev. Biochem.* 82, 171–202. (2) Ito, K., Chadani, Y., Nakamori, K., Chiba, S., Akiyama, Y., and Abo, T. (2011) Nascentome analysis uncovers futile protein synthesis in *Escherichia coli*. *PLoS ONE* 6(12): e28413.

P657**Board Number: B909****The ERAD lectin OS-9 facilitates turnover of non-native, hyper-glycosylated forms of the ER HSP90, GRP94.**D. Dersh^{1,2}, S.M. Jones³, D. Eletto², J.C. Christianson³, Y. Argon²;¹Biochemistry and Molecular Biophysics, University of Pennsylvania, Philadelphia, PA, ²The Children's Hospital of Philadelphia, Philadelphia, PA, ³Ludwig Institute for Cancer Research, University of Oxford, Oxford, United Kingdom

In order to produce properly folded secretory, transmembrane, and cellular proteins, quality control (QC) pathways in the endoplasmic reticulum (ER) rely on efficient synergy between protein folding and disposal mechanisms. Terminally misfolded or orphaned polypeptides can be cleared via ER-associated degradation (ERAD), a process that involves substrate recognition, retrotranslocation across the lipid bilayer to the cytosol, and proteasomal degradation. While some ERAD-initiating signals have been uncovered (for instance the rate limiting trimming of mannose residues on N-linked glycans), little is known about the coupling of pro-folding components with ERAD machinery during protein QC. We hypothesized that the ER-resident HSP90 family member GRP94 could play a role in this crosstalk because it is a well-documented molecular chaperone that also associates robustly with OS-9, a lectin involved in the substrate recognition phases of ERAD. Unexpectedly, we discovered that GRP94 does not participate with OS-9 in the degradation of misfolded substrates, nor does it interfere with OS-9's capacity to recognize and clear ERAD targets. Instead, the GRP94/OS-9 complex forms because OS-9 preferentially associates with a subpopulation of GRP94 marked by hyper-glycosylation. Molecules of GRP94 that are aberrantly modified on cryptic N-linked glycan acceptor sites have a non-native conformation and lower activity, rendering them biologically unstable. OS-9 sequesters these species and assists in their disposal via an ERAD-independent, lysosomal-like mechanism. Therefore, the GRP94/OS-9 complex is important not for coordination in ERAD but rather to trap and degrade a pool of malformed chaperone. This highlights a novel mode of protein regulation, whereby glycosylation of cryptic acceptor sites dictates the function and fate of a typically long-lived ER-resident chaperone.

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P658**Board Number: B910****PERK activation interferes with myelin formation and signals in a mouse model of CMT1B.**M. Sidoli^{1,2}, N. Musner³, D.R. Cavener⁴, M. Feltri⁵, L. Wrabetz⁵;¹Biochemistry, University at Buffalo, Buffalo, NY, ²Hunter James Kelly Research Institute, Buffalo, NY,³San Raffaele Scientific Institute, Milan, Italy, ⁴Pennsylvania State University, University Park, PA,⁵Biochemistry and Neurology, University at Buffalo, Buffalo, NY

Schwann cells (SCs) are the myelinating glial cells of the peripheral nervous system. To form functional myelin, SCs produce an enormous amount of proteins and lipids, and the quality control of the myelin proteins is highly regulated. Mutations in myelin genes result in a variety of inherited neuropathies and a wide range of phenotypes. Myelin Protein Zero (MPZ, P0), is a type I transmembrane protein abundantly produced by myelin-forming SCs. Deletion of serine 63 from P0 (POS63del) causes Charcot-Marie-Tooth type 1B (CMT1B) demyelinating neuropathy both in humans and mice. POS63del accumulates in the endoplasmic reticulum (ER) of SCs where it elicits an unfolded protein response (UPR). PERK, ATF6 and IRE1 UPR transduction pathways are active in nerves of POS63del mice. In particular, PERK phosphorylates the translation initiation factor eIF2 α , which attenuates global translation and reduces ER stress. In fact, *Perk* ablation specifically in POS63del Schwann cells (*S63del//Perk^{cko}*) decreased the level of phospho-eIF2 α compared to S63del nerves. Moreover, extensive accumulation of P0 misfolded protein remains in the ER of SCs. Nonetheless, conditional ablation of *Perk* paradoxically improves the demyelinating phenotype of POS63del mice in vivo and in myelinating explant cultures. We therefore investigated the hypothesis that *Perk* ablation may provoke a compensatory hyperactivation in the other two arms of the UPR, ATF6 or IRE1. However, in *S63del//Perk^{cko}* compared to S63del nerves neither cleaved ATF6 and its target chaperones, nor spliced Xbp1 mRNA levels were increased. These data suggest that PERK may interfere with signals outside of the UPR. We have preliminary data that PERK inhibits a target in a pro-myelinating pathway. This finding would be consistent with the notion that the UPR participates in physiological processes not related to unfolded or misfolded proteins.

P659**Board Number: B911****Identifying structural elements within the ENaC α subunit required for chaperone-dependent ER-associated degradation (ERAD).**T.M. Buck¹, A. Jordahl¹, E. Cook¹, J.L. Brodsky²;¹Biological Sciences, Univ Pittsburgh, Pittsburgh, PA, ²Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA

The epithelial sodium channel, ENaC, is responsible for the reabsorption of sodium in the kidney collecting duct. ENaC is a heterotrimer, composed of homologous α -, β - and γ -subunits. Each subunit contains two transmembrane segments, a large extracellular domain, and short cytosolic N- and C-termini. Gain-of-function mutations in ENaC lead to high blood pressure and Liddle's syndrome, whereas loss-of-function mutations lead to low blood pressure, salt-wasting, and Pseudohypoaldosteronism Type1. We and others have shown that unassembled ENaC subunits and even a significant fraction of the assembled channel are recognized by the quality control machinery in the endoplasmic reticulum (ER), and targeted for ER-associated degradation (ERAD). The ERAD machinery recognizes proteins that fail to properly fold or assemble and selects them for degradation by the cytosolic 26S proteasome. In order to characterize the ERAD requirements for ENaC, we have employed a yeast expression system. Using this system we recently determined that the ER molecular chaperone, Lhs1, which is a GRP170 homolog, is required for targeting α ENaC for ERAD, but is dispensable for degrading the β - or γ ENaC subunits (Buck et al., JBC, 2013).

To identify the structural elements that are unique to α ENaC and are required for Lhs1-dependent degradation, we constructed a series of chimeric ENaC subunits and tested their stabilities in wild type and mutant yeast strains. Because only the C-terminus of the α subunit harbors an ER exit site, we initially hypothesized that this and perhaps other elements within the C-terminus of α ENaC were necessary for Lhs1-dependent degradation. We determined that the C-terminal region of α ENaC is at least partially responsible for Lhs1-targeted ERAD. Replacing the C-terminus of α ENaC with the C-terminus of either β - or γ ENaC reduces Lhs1-dependent degradation, and replacing either the β - or γ ENaC C-terminus with that of α ENaC changes the Lhs1-dependent degradation profile. However, the glycosylation state and extracellular domains are also likely playing a role in Lhs1-dependent ERAD targeting. Ongoing studies are investigating the role of the large extracellular/ER lumenal loops in Lhs1-dependent degradation. This work is supported by National Institute of Health grants DK090195 to T.M.B., GM75061 to J.L.B., and DK79307 (University of Pittsburgh George O'Brien Kidney Research Center).

P660**Board Number: B912****The degradation requirements for topologically distinct quality control substrates in the yeast endoplasmic reticulum.**C.J. Guerriero¹, A.A. Augustine¹, J.L. Brodsky¹;¹Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA

In eukaryotic cells, nearly one third of all newly synthesized proteins are translocated into the endoplasmic reticulum (ER). Inside the ER, proteins are post-translationally modified and folded by molecular chaperones into their native states. Mutations, transcriptional or translational errors, or failure to obtain the proper post-translational modifications can result in protein misfolding. A quality control pathway, ER-associated degradation (ERAD), recognizes and degrades misfolded proteins to prevent toxic gain-of-function aggregates. Given the vast array of conformers that proteins may adopt, it is not surprising that many diseases result from protein misfolding or protein aggregation, which cannot be averted by ERAD.

The rate-limiting step during ERAD is substrate retrotranslocation from the ER lumen into the cytoplasm, which occurs prior to 26S proteasome-mediated degradation. Therefore, the retrotranslocation step can tune degradation efficiency to mitigate ERAD-related diseases. Retrotranslocation is energetically driven by Cdc48p, which extracts integral membrane proteins either directly from the ER membrane or through an as-yet-elusive retrotranslocation channel. Integral membrane proteins have diverse topologies with varying degrees of exposure to the cytoplasmic or luminal faces of the ER. However, little is known about how the topology of an ERAD substrate influences ERAD efficiency. We predict that extraction of a large ER luminal domain may slow retrotranslocation kinetics.

To test this hypothesis, a pair of model ERAD substrates were designed to contain the same truncated nucleotide-binding domain from a well-characterized ERAD substrate, Ste6p, *but on opposite sides of the ER membrane. We previously demonstrated that a soluble version of this domain, NBD2, when expressed as a cytoplasmic protein is degraded by the proteasome, and therefore can be used as a transposable degenon* (Guerriero *et al.*, *JBC*, 2013). *The first model substrate, Chimera A, is a dual pass membrane protein fused to NBD2 exposed to the cytoplasm. By contrast, Chimera γ , is a single pass membrane protein which deposits NBD2 in the ER lumen.* Initial characterization confirms the proper topology of these substrates and their proteasome-dependent degradation. Moreover, the substrates rely on ER resident E3 ligases, Hrd1p and Doa10p, for efficient degradation. We are currently determining the relative dependence on Cdc48p for both degradation and retrotranslocation in order to better understand the energetic barriers to ERAD (Supported by NIH grants DK101584 to C.J.G. and GM075061 to J.L.B.).

P661**Board Number: B913****Potentiated Hsp104 Variants Antagonize Diverse Proteotoxic Misfolding Events.**M.E. Jackrel¹, M.E. DeSantis¹, B.A. Martinez², L.M. Castellano¹, R.M. Stewart³, K.A. Caldwell², G.A. Caldwell², J. Shorter⁴;¹Biochemistry and Biophysics, University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA, ²University of Alabama, Tuscaloosa, AL, ³Department of Cell Biology, Yale University, New Haven, CT, ⁴Department of Biochemistry and Biophysics, Perelman School of Medicine University of Pennsylvania, Philadelphia, PA

There are no therapies that reverse the proteotoxic misfolding events that underpin fatal neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD). Treatments for these disorders remain palliative, and no therapeutics exist that address their underlying cause. However, not all amyloidogenic events are associated with toxicity. In yeast, amyloids are utilized for beneficial purposes and thus tightly regulated. Hsp104, a conserved hexameric AAA+ protein from yeast, solubilizes disordered aggregates and amyloid but has no metazoan homolog and only limited activity against human neurodegenerative disease proteins. We have reengineered Hsp104 to rescue TDP-43, FUS, and α -synuclein proteotoxicity by mutating single residues in the middle domain or the small domain of nucleotide-binding domain 1. Potentiated Hsp104 variants enhance aggregate dissolution, restore proper protein localization, suppress proteotoxicity, and in a *C. elegans* PD model attenuate dopaminergic neurodegeneration. Potentiating mutations reconfigure how Hsp104 subunits collaborate, desensitize Hsp104 to inhibition, obviate any requirement for Hsp70, and enhance ATPase, translocation, and unfoldase activity. Furthermore, these variants suppress the toxicity of disease-associated missense mutant versions of TDP-43, FUS, and α -synuclein. Our work establishes that disease-associated aggregates and amyloid are tractable targets and that enhanced disaggregases can restore proteostasis and mitigate neurodegeneration.

P662**Board Number: B914****Subcellular Localization of Heat Shock Factor 1 Alpha and Beta Isoforms.**K. Hassman¹, J.R. Williams¹, N.J. Bachman¹;¹Biology Department, SUNY Oneonta, Oneonta, NY

Heat shock causes proteins to denature or unfold which alters their function in the cell. Heat shock transcription factor 1 (HSF1) is responsible for the transcriptional response to produce heat shock proteins to protect cells from induced stress. Research is being conducted on different isoforms of HSF1, predominantly alpha and beta isoforms. Isoforms are versions of the protein that are similar, but not identical, and they may serve distinct functions. The goal of this research is to discover the subcellular localization pattern of HSF1 alpha and beta isoforms in the presence or absence of heat shock. The experimental approach involved growing NIH 3T3 cells on fibronectin coverslips. Separate dishes were

transfected with expression plasmid DNA encoding the isoform. Coverslips were then incubated with primary (anti-HA-tag) and fluorescent secondary antibody (conjugated to green Alexa 488), and viewed using fluorescent microscopy to visualize localization of the isoforms in NIH 3T3 cells. We have observed a different pattern of localization for the alpha and beta isoforms. HSF1 beta seems to be mostly localized in the nucleus both before and after heat shock. HSF1 alpha is distributed in both the cytoplasm and the nucleus, although more nuclear than cytoplasmic staining is seen. Following heat shock; there is an even lower proportion of cytoplasmic staining for HSF1 alpha. To test the effects on isoform distribution after disrupting nuclear import, nuclear localization signal (NLS) mutants of the HSF1 alpha isoform tagged with HA were constructed by overlap extension PCR. Essentially all fluorescent staining of HA was found in the cytoplasm, consistent with previous studies. We are currently constructing putative NLS mutants of HSF1 beta to see if the beta isoform is imported by the same pathway. This work was supported by NIH NIGMS grant R15 GM096231 to N. J. B.

P663

Board Number: B915

Adaptations in the proteostasis network of *Dictyostelium discoideum* cause an unusual resilience to protein aggregation.

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Many protein-misfolding diseases are caused or exacerbated by proteins carrying prion-like domains, which are sequences of low complexity enriched for polar amino acids, such as asparagine and glutamine. These sequences are called 'prion-like', because they show extensive sequence similarity to proteins that undergo structural conversions from an intrinsically disordered to an aggregated prion state, which often conveys self-propagating capabilities. Using a bioinformatic algorithm for the identification of prion-like sequences, we screened the proteome of the social amoebae *Dictyostelium discoideum*. We find that *D. discoideum* has the highest content of prion-like proteins of all organisms investigated to date, suggesting that its proteome has a high overall aggregation propensity. To study mechanisms involved in the regulation of these proteins, we analyzed the behavior of several well-characterized prion-like proteins from other organisms, such as an expanded version of the exon 1 of human huntingtin (Q103) and the prion domain of the yeast prion protein Sup35 (NM) in *D. discoideum*. We find that these proteins remain soluble in the cytosol and are innocuous to *D. discoideum*, in contrast to observations in other organisms, where the same proteins form cytotoxic cytosolic aggregates. However, when exposed to heat stress or drugs that inhibit molecular chaperones, these proteins aggregate and become cytotoxic, suggesting a major role for molecular chaperones in the regulation of prion-like proteins. We further show that the disaggregase Hsp101, a molecular chaperone of the Hsp100 family, dissolves heat-induced aggregates of prion-like proteins and promotes thermotolerance. Additional findings indicate that the nucleus serves as a compartment for protein quality control, where prion-like proteins are targeted for degradation by the ubiquitin-proteasome system. Taken together, our data suggest that the protein quality control system of *D. discoideum* has

undergone specific adaptations that increase the proteostatic capacity of this organism and allow for an efficient regulation of its aggregation-prone prion-like proteome.

P664

Board Number: B916

Ameliorating potentiated Hsp104 variants.

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Hsp104 is a hexameric, AAA+ protein disaggregase with no metazoan homologue found in *S. cerevisiae*. Hsp104 refolds protein aggregates, resolubilizes prefibrillar oligomers and amyloid, and is an attractive agent for combatting protein misfolding underlying many neurodegenerative diseases. We previously engineered hyperactive Hsp104 variants that potently suppress toxicity from disease substrates TDP-43, FUS, and α -synuclein, and are not overly proteotoxic to yeast. This enhanced activity would be best honed against a specific disease substrate to avoid nonspecific unfolding of proteins. We hypothesized that by introducing subtle changes in conserved tyrosine residues in the substrate-binding pore loops of Hsp104, we could engineer potentiated variants to be selective for a single disease substrate. We used an overexpression system with a galactose-inducible promoter to explore a wide range of potentiated variants. In using several different backgrounds containing activating mutations as starting platforms to rationally introduce specific mutations, we found hyperactive variants capable of strongly suppressing toxicity from all three disease substrates. We additionally uncovered several variants that seem to selectively rescue α -synuclein toxicity, thus enhancing the substrate specificity of Hsp104.

P665

Board Number: B917

Adenosine Receptors as Modulators of the Cellular Stress Response.

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Both inhibitory and stimulatory adenosine receptors are aberrantly expressed in the tissue of patients with neurodegenerative diseases, but the exact role that these receptors play in the progression of disease is not clear. While previous data from our lab has indicated that expression of inhibitory receptor A1R plays a role in modulation of SIRT1 activity, other research implicates A1R in activation of the inflammatory pathways. We hypothesize that during oxidative stress, a common pathway in neurodegeneration, adenosine receptor expression modulates epigenetic factors, such as SIRT1, to

activate the cellular stress response pathways. Thus, these receptors may play a role in controlling the stress response and ultimately cell survival. Using immunoblot, ELISA and mitochondrial function assays, we will demonstrate the role that adenosine receptors play in controlling the stress response, particularly in the presence of reactive oxygen species. We will show a change in receptor localization following oxidative stress, as well as an increase in SIRT expression as a result of increased receptor expression.

P666

Board Number: B918

Involvement of ER stress and Ca²⁺ signaling in colitis-associated damage in intestinal epithelial cells.

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Ulcerative colitis (UC) can extend to the terminal ileum through the influx of colonic content via an inflamed ileocecal valve. Under these circumstances, gut mucosa integrity and functions are altered and may contribute to the pathogenesis of UC. The endoplasmic reticulum (ER) has a crucial role in the synthesis, correct folding and sorting of proteins, and is the main calcium (Ca²⁺) reservoir in the cell. A chronic decrease in ER Ca²⁺ concentrations can lead to misfolded proteins and eventually to ER stress. The aim of this work is to study the role of Ca²⁺ signaling in ER stress, a hallmark of UC, and how this is linked to the dysfunction of the intestinal epithelial cells. A mixture consisting of the cytokines IL-1 β -, MCP1, and TNF- α was used to induce ER stress in Caco-2 cells 0, 3 and 7 post- confluency. ER stress induction in Caco-2 cells was tested by measuring the levels of chaperone proteins. Interestingly, we found that the stress markers BiP and Chop were significantly increased 8 and 24 hours after the cytokine treatment. Moreover, the activity of sucrase-isomaltase, a major glycoprotein of the intestinal brush border membrane, significantly decreased after 8 and 24 hours of treatment of 3 and 7 days post-confluent cells. Furthermore, transepithelial resistance (TEER) was significantly decreased after treatment at 8, 24 and 48 hours. The results to date suggest that ER stress is indeed induced by the mixture of the cytokines utilized and that the epithelial barrier integrity is affected. Ongoing experiments will focus on studying the link between ER stress and Ca²⁺ signaling to gain insights into the mechanisms that affect the activity of intestinal brush border enzymes in UC.

P667**Board Number: B919****Increased Expression of the Large Conductance, Ca²⁺ Activated and Voltage-Dependent (BK) Channel Represents an Early Event During Neurodegeneration.**J. Braun¹, J. Donnelier¹, B. Kyle², C. Koutras¹, E. Ahrendt¹;¹Biochemistry and Molecular Biology, Univ Calgary/Hotchkis Brain Institute, Calgary, AB, ²Univ Calgary/Libin Brain Institute, Calgary, AB

We have previously shown that in the CSP α KO mouse model of neurodegeneration, there is a 2.5 fold increase in the expression of the large conductance, Ca²⁺ activated and voltage-dependent (BK) channel. Functionally, neuronal BK channels are activated by both membrane depolarization and elevated intracellular Ca²⁺ levels and are central to the regulation of action potential duration and neurotransmitter release. CSP α (cysteine string protein) null mice have age-dependent and activity-dependent synapse loss and neurodegeneration. CSP α is a synaptic vesicle-associated molecular chaperone. The cellular chaperone network maintains protein homeostasis and cellular function and imbalances in proteostasis may trigger pathogenic cascades that lead to neurodegeneration. CSP α is essential for synapse maintenance. Mutations in CSP α cause adult neuronal ceroid lipofuscinosis (ANCL) a rare neurodegenerative disease.

Here we evaluate the time course of expression changes in BK channel in the severe mouse model of Alzheimer's disease (5XFAD). 5XFAD transgenic mice overexpress mutant human amyloid precursor protein (APP(695)) with the Swedish (K670N, M671L), Florida (I716V), and London (V717I) mutations as well as human presenilin1 (PS1) harboring, M146L and L286V that cause familial Alzheimer's disease. Degeneration, neuron loss and amyloid deposits start at about 3 months, A β 42 accumulates and memory is impaired recapitulating pathologic and behavioral characteristics of Alzheimer's disease. Like the CSP α knock out mouse model, synapse loss is characteristic of 5XFAD mice. However, in contrast, the impairment of synaptic function in 5XFAD mice develops later (i.e. post natal 3 months (5XFAD) vs 3 weeks (CSP α KO)).

Our latest results suggest that elevation of BK channel levels represent an early event in the onset of neurodegeneration. Impairment of CSP α activity leads to dysregulation of BK channel levels in these neurodegenerative mouse models. Together these findings provide new insight into the possibility of re-purposing therapeutic agents that target ion channels for the treatment of neurodegenerative diseases. We conclude that CSP α -associated changes in BK channel levels may contribute to the pathogenic cascade of events leading to neurodegeneration.

P668**Board Number: B920****Ixeris dentata induces regulation of amylase secretion and inhibits Endoplasmic Reticulum stress in Streptozotocin induced diabetic rats.**K. Bhattarai¹, H. Kim², H. Chae^{3,4};

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Ixeris dentata, is an herbal plant which is generally used for the treatment of liver diseases, diabetes and to improve appetite in Korea and China. ER stress is activated in various tissues under conditions related to obesity and diabetes. Conditions such as hyposalivation and xerostomia also can alter the protein folding capacity and cause ER stress. The objectives of this study were to investigate the effects of IXD extract in streptozotocin induced diabetic rats and to improve the salivation rate on dry mouth condition induced by diabetes. Type 1 diabetes was induced by a single intraperitoneal injection of freshly prepared STZ (65mg/kg), and 5% IXD extract (100mg/kg) was administered orally for 10 days to observe the effect. Treatment with extracts of IXD resulted in a significant increase in salivary flow rate and also have increased the salivary gland weight in IXD treated diabetic rats. IXD also reduced the total protein concentration in saliva of diabetic rats. The amylase activity was also increased in IXD treated diabetic rats in both saliva as well as in gland lysates. Immunohistochemistry data proved this result and has shown more secretion of alpha amylase in IXD treated diabetic rats. IXD improved the morphology of sub mandibular serous acinar cells in treated rats. Moreover, we checked whether IXD extract inhibits ER stress, and found the increased ER stress response in diabetic control rats and was inhibited by the treatment of IXD extracts. The decreased expression of GRP78, and XBP-1 shows the inhibitory effect of IXD extracts. So, this study revealed the potential value of the IXD extract for the treatment of diabetes or its resultant condition such as xerostomia and ER stress. Key words: IXD, ER stress, Xerostomia,

Chemical Biology

P669

Board Number: B922

Protein expression profile of human insulin secretory granules mapped by 2D-DIGE and 1D SDS-PAGE coupled with nanoLC-ESI-MS/MS bottom-up proteomics.

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Insulin secretory granules (ISGs) are crucial organelles of pancreatic β -cells and represent a key participant to glucose homeostasis. Insulin is packed and processed within these vesicles before its release by exocytosis. It is therefore vital to acquire qualitative and quantitative data on the ISG proteome, in order to increase our knowledge on ISG biogenesis, maturation and exocytosis. Many efforts have been made in the past years to increase the coverage of the ISG proteome. However, much of the existing work on the composition of granules was done in immortalized cell lines or rodent tissues which may not faithfully reflect the composition of granules in human islets. The major goal of the current research was to characterize the proteome of an enriched fraction of ISGs from the human beta cells. Herein, we present the global proteomic profiling of human ISGs using two analytical methodologies coupled with nanoliquid chromatography-tandem mass spectrometry: one-dimensional gel electrophoresis (1DEF nanoLC Orbitrap-ESI-MS/MS), and two-dimensional fluorescence difference-in-gel electrophoresis (2D-DIGE nanoLC-ESI-MS/MS). The 253 significantly identified proteins (p

P670

Board Number: B923

One Step Detection of Oxidative Stress-Induced Carbonylation in Live Mammalian Cells.

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Carbonylation of proteins, lipids and DNA is a common manifestation of oxidative stress. Due to the involvement of oxidative stress in disease conditions, such as neurodegenerative diseases and diabetes, cellular carbonylation is considered a biomarker in understanding the etiology and progression of these diseases. Presently used methodologies for detecting intracellular carbonylated biomolecules require fixing or lysing the cells for lengthy downstream processing. Here, we present two hydrazine-functionalized synthetic fluorophores (coumarin hydrazine and benzocoumarin hydrazine) that permit a single step detection of biomolecule-carbonyls in live cells. When administered to the cells, these fluorophores readily react with biomolecule-carbonyls; the resultant hydrazone products exhibit a shift

in absorption and emission maxima, and an increase in quantum yield. These photochemical properties of the fluorophore allow the detection of the products in the presence of unreacted fluorophore, thereby obviating the necessity of removing excess detection reagent. Additionally, these fluorophores are non-toxic. Therefore we report that a one step detection method, involving a brief incubation of the cells with the hydrazine-fluorophore before microscopic analysis, enables visualization of carbonylated biomolecules in live cells. We demonstrated the applicability of these fluorophores in PC3 and A549 cell lines. Hydrogen peroxide- and serum starvation-induced biomolecule carbonylation were conveniently detected by these coumarin based fluorophores. This one step method for detecting oxidative stress induced carbonyls in live cells should be applicable for high content screening for inducers of intracellular carbonylation.

P671

Board Number: B924

Aptamer-gated silica nanoparticles synthesized via click chemistry.

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Precise targeting of drug loaded nanocapsules to the diseased locations is a challenging task. Aptamer technology provides viable alternative to overcome the present limitations with specific cell targeting. Aptamer-gated nanocapsules have been developed as drug delivery system with dual function of biorecognition and molecular gating. In this study, mesoporous silica nanoparticles were grafted with amino-silane and used for immobilization of aptamers via click chemistry. Synthesized particles were characterized by DLS, Fourier transform infrared spectroscopy (FTIR), and transmission scanning electron microscopy (TEM). The aptamers were alkyne-functionalized for conjugation with azide-modified silica particles via click chemistry reaction. The kinetic constants (K_m and V_{max}) were determined by measuring initial reaction rates using the artificial chromogenic substrate. Fluorescence properties of synthesized aptamer-gated nanoparticles were also monitored before/after click reaction with the fluorophore. Kinetics of the release of loaded Cargo were determined and compared to aptamer-gated particles synthesized via traditional conjugation chemistry techniques.

P672**Board Number: B925****Efficient phosphate-catalyzed hydrazone reaction with improved biocompatibility for bioorthogonal conjugations.**O. Dilek¹, S.L. Bane², A.M. Sorrentino²;¹School of Medicine, Istanbul Kemerburgaz Univ, Istanbul, Turkey, ²Chemistry, SUNY-Binghamton, Binghamton, NY

Bioorthogonal site-specific chemical reaction to label biomolecules in vitro and in living cells is one of the most powerful tools in chemical biology. A reactive pair frequently used for chemical conjugation is aldehydes/ketones with hydrazines/hydrazides/oximes. Although the reaction is generally specific for the two components, even in a cellular environment, the reaction is very slow under physiological conditions. Additives such as 10 mM aniline are often used to increase the reaction rate, but these chemicals can be detrimental to the biological system under investigation. We have found that adding an ortho-phosphate group to an aromatic aldehyde increases the reaction rate with a hydrazine or hydrazide by an order of magnitude. An added benefit is that phosphate enhances the aqueous solubility of the reagent and its hydrazone product. We have synthesized phosphate-substituted aldehyde synthetic models to study kinetics of their reactions with hydrazines and hydrazides that contain a fluorophore. Fluorescence properties of hydrazone products are also investigated. This rapid bioorthogonal reaction should therefore be potentially a very useful strategy for routine site-specific chemical ligations to study and image complex cellular processes in biological systems.

P673**Board Number: B926****GSK-3 modulates cellular responses to a broad spectrum of kinase inhibitors.**C.A. Thorne¹, C. Wichaidit¹, A.D. Coster¹, B.A. Posner², L.F. Wu³, S.J. Altschuler³;¹Green Center for Systems Biology, University of Texas Southwestern Medical Center, Dallas, TX,²Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX, ³Department of Pharmaceutical Chemistry, University of California, San Francisco, San Francisco, CA

A fundamental challenge in treating disease is identifying molecular states that affect cellular responses to drugs. Here, we focus on GSK-3, a key regulator for many of the hallmark behaviors of cancer cells. We alter GSK-3 activity in colon epithelial cells to test its role in modulating drug response. We find that GSK-3 activity broadly affects the cellular sensitivities to a panel of oncology drugs and kinase inhibitors. Specifically, inhibition of GSK-3 activity can strongly desensitize or sensitize cells to kinase inhibitors (e.g. mTOR or PLK1 inhibitors, respectively). Additionally, colorectal cancer cell lines, in which GSK-3 function is commonly suppressed, are resistant to mTOR inhibitors and yet highly sensitive to PLK1 inhibitors and this is further exacerbated by additional GSK-3 inhibition. Finally, by conducting a kinome-wide RNAi screen, we find that GSK-3 modulates the cell proliferative phenotype of a significant fraction (~35%) of the kinome, which includes ~50% of current, clinically relevant kinase-targeted drugs. Our results

highlight an under-appreciated interplay of GSK-3 with therapeutically important kinases and suggest strategies for identifying disease-specific molecular profiles that can guide optimal selection of drug treatment.

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Board Number: B927

Endogenous reactive oxygen species in α -syntrophin knock-downed cells hindered myoblast differentiation.

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α -Syntrophin is a component of dystrophin-glycoprotein complex which interacts with various intracellular signaling proteins in muscle cells. To study on the function of α -syntrophin in muscle cell, α -syntrophin knock-downed C2 cell line was established by infecting lentivirus particles with α -syntrophin shRNA. Muscle differentiation is a multi-step process including the expression of muscle regulatory factors, cell cycle arrest and cell fusion into multinuclear myotubes. The α -syntrophin knock-downed cells (SNKD), however, were characterized by defect of terminal differentiation and reduction of cell fusion index. Myogenesis is also importantly accompanied by an intensive mitochondrial biogenesis. Since the amount of reactive oxygen species (ROS) correlates with mitochondrial content, the generation of intracellular ROS is also inescapably increased during myogenesis. To confirm an excessive accumulation of intracellular ROS during differentiation of SNKD cells, mitochondrial H₂O₂ imaging was performed using two-photon microscopy. The result shows that mitochondrial H₂O₂ in SNKD myotubes increased than that of control shRNA transfected cells. Epigallocatechin-3-gallate (EGCG) is a well-known ROS scavenger as well as an antioxidant chemical. By using differentiation media containing EGCG, a reduction of fusion index in SNKD cells was dramatically recovered. It demonstrates that intracellular ROS produced during myogenesis hinders terminal differentiation of SNKD cells. In addition, the activity of antioxidant enzymes is changed during myoblast differentiation, which enables myotubes to modulate the susceptibility to oxidative stress. We found that the expression of catalase was substantially decreased during differentiation of SNKD cells. These results suggest that α -syntrophin knock-downed cells have a low capacity for antagonizing oxidative stress endogenously generated during myogenic differentiation.

P675**Board Number: B928****The Protective Effects of J-10 in Preventing Cell Proliferation and Migration of Vascular Smooth Muscle Cells in Balloon Injury-Induced Neointimal Hyperplasia.**P. Li^{1,2}, C. Wu³;¹PHARMACY, China Medical University, Taichung, Taiwan, ²Pharmacy, University of Southern California, Los Angeles, CA, ³Pharmacy, Taipei Medical University, Taipei, Taiwan

Restenosis after balloon angioplasty procedures is a significant clinical problem. The major biological process of restenosis are proliferation and migration of vascular smooth muscle cells (VSMCs). The compound J-10, which is isolated from marine coral, has shown antioxidant activity, but has not been reported to have an anti-restenosis effect. The growth inhibitory effect of J-10 on VSMCs were determined by MTT assay. The mechanism analysis of migration were determined by using a wound healing assay, a transwell assay, gelatinase zymography, and western blotting. The effect of J-10 on restenosis were accessed by the ratio of the neointima-to-media area (N/M ratio). The results showed that J-10 reduced neointima formation in the artery of rats. J-10 significantly inhibited VSMCs proliferation and migration through FAK, PI3K, AKT, and ERK1/2 pathway, as shown by western blot. In addition, enzymatic action of matrix metalloproteinases 2 and 9 (MMP-2 and MMP-9) was carried out by gelatinase zymography. The compound J-10 decreased the expression levels of MMP-2 and MMP-9. This is the first study to show the compound J-10 prevented balloon angioplasty-induced neointimal hyperplasia as well as VSMCs proliferation and migration both *in vivo* and *in vitro*. Thus, J-10 may be a novel useful anti-restenosis agent.

P676**Board Number: B929****Structure-activity relationship study for integrin $\alpha 6\beta 1$ -binding peptide A2G10 derived from mouse laminin $\alpha 2$ chain sequence.**F. Katagiri¹, R. Yamanaka¹, R. Suyama¹, N. Nakajima¹, Y. Yamada¹, K. Hozumi¹, Y. Kikkawa¹, M. Nomizu¹;¹School of Pharmacy, Tokyo Univ Pharm/Life Sci, Hachioji, Japan

Laminins are major components of basement membrane, consists of three different subunits, α , β , and γ chains, and so far, five α , three β , and three γ chains have been identified. We have constructed the synthetic peptide library derived from the laminin sequences, and identified cell adhesive peptides. We have reported that A2G10 peptide (SYWYRIEASRTG, mouse laminin $\alpha 2$ chain, 2223-2234) interacts with integrin $\alpha 6\beta 1$, and promotes strong cell adhesion [1]. Recently, integrin $\alpha 6\beta 1$ is suggested to play an important role for stem cells to maintain their functions [2]. In this study, we investigated the structure-activity relationship of A2G10 to identify the specific motif for integrin $\alpha 6\beta 1$. The truncated peptides of A2G10 (A2G10Nd1--4, A2G10Cd1--4) were synthesized by Fmoc-solid phase methods, and evaluated their human dermal fibroblast (HDF) attachment activities. The A2G10-derived peptides which were

deleted Ser1 (A2G10Nd1) from N-terminal and Arg10-Thr11-Gly12 from C-terminal (A2G10Cd3) showed HDF attachment activity. From the result we designed A2G10min peptide (YWYRIEAS, mouse laminin α 2 chain, 2224-2231). A2G10min promoted HDF attachment, which was inhibited by EDTA, anti-integrin α 6, and β 1 antibodies. Next, the peptides substituted each amino acid residue for alanine (A2G10A1--A6, and A8) or glycine (A2G10minG1--G8) were prepared, and evaluated their HDF attachment activities (Ala and Gly scan). The peptides substituted Arg4 and Ser8 for Ala (A2G10minA4 and A8) maintained HDF attachment activities. The peptides substituted Ala7 and Ser8 for Gly (A2G10minG7 and G8) maintained HDF attachment activities. The result of Ala scan suggested that Arg4 and Ser8 residues of A2G10min were not important for integrin α 6 β 1 binding. From the result of Gly scan, we designed A2G10min-derived peptide A2G10minX (YWYRIEGG) and its C-terminus truncated peptides A2G10minY (YWYRIEG) and Z (YWYRIE). A2G10minX, Y, and Z promoted HDF attachment, which was inhibited by EDTA, anti-integrin α 6, and β 1 antibodies. We concluded that the core motif of A2G10 to interact with integrin α 6 β 1 was YWYRIE (mouse laminin α 2 chain, 2224-2229).

[1] Urushibata S, Hozumi K, Ishikawa M, Katagiri F, Kikkawa Y, Nomizu M. Identification of biologically active sequences in the laminin alpha2 chain G domain. Arch Biochem Biophys. 2010;497:43-54. [2] Rodin S, Domogatskaya A, Strom S, Hansson EM, Chien KR, Inzunza J, et al. Long-term self-renewal of human pluripotent stem cells on human recombinant laminin-511. Nat Biotechnol. 2010;28:611-5.

P677

Board Number: B930

Assembling proteins with modular DNA scaffolds: a strategy for engineering antibodies and probing cellular signaling complexes.

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An antibody's class is determined by its constant region, or scaffold, which encodes the valency, effector functions, and higher-order architecture of the variable domains. *In vivo*, antibodies of different classes serve specialized roles during the immune response and have different biological activities. Expansion of scaffold diversity has the potential to generate enhanced therapeutics with properties that include increased avidity, increased specificity, and the ability to recognize specific combinations of receptors on cells or deliver protein-based effectors. However, systematic exploration of antibody scaffold geometry, valency, and combinatorial binding capacity is difficult with protein-based scaffolds due to the challenges associated with protein design. Instead, we are using DNA-based scaffolds, which are modular, programmable and can control the position and orientation of pendant proteins with nanometer resolution. This strategy requires a simple and modular technique for site-specific conjugation of synthetic oligonucleotides to proteins. Our approach uses the aldehyde tag, which can be genetically incorporated into proteins expressed in both bacterial and mammalian expression systems.

We designed and optimized four different reactions that generate DNA–protein conjugates to provide flexibility in linker chemistry. Using this strategy, we conjugated oligonucleotides to a suite of antibody fragments, including an N-terminal tagged Fab, a C-terminal tagged Fc, and an internally-tagged IgG. Using SDS-PAGE and transmission electron microscopy, we demonstrated that the protein-DNA conjugates can be efficiently assembled into dimeric and trimeric structures. Our DNA-scaffolded antibodies bind their targets on live cells and can be hierarchically assembled into structures of greater valency and complexity. We are applying these techniques to develop bispecific antibodies targeting cell surface proteins on a triple negative breast cancer cell line. Additionally, we are using these modular DNA scaffolds to investigate the effect of nanoscale organization on the signaling profiles of receptor tyrosine kinases by systematically guiding the assembly of signaling complexes on cells.

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Board Number: B931

Treatment of Hydraulic Fracturing Contaminated Water using *Closterium moniliferum*: Growth rate, nuclear integrity, and Ba elemental analysis of algal biomass.

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The acquisition of metals is essential for a variety of protein and cellular functions, including sensing of environmental conditions. To sense gravitational force, some algae actively accumulate metal sulfates to cellular concentrations well above the surroundings' concentration. The single-celled green algae *Closterium moniliferum* selectively accumulates approximately 1 picogram of the toxic, heavy metal barium (Ba) per cell after a 24 hour period, which is then incorporated as barium sulfate crystals as statoliths in the terminal vacuoles. Thus, *C. moniliferum* may be used to contain accidental release of Ba into the environment from industrial activities, such as hydraulic fracturing. Hydraulic fracturing technology collects natural gas through the injection and subsequent collection of hydraulic fracturing fluid into and from shale rock. In addition to gas absorption, fracking fluid dissolves Ba containing minerals and upon recovery contains a Ba concentration of thousands of milligrams per liter. *C. moniliferum* is an attractive candidate for phycoremediation of Ba from mutagenic, biocidal fracking contaminated water because of its robust DNA repair system. To further assess its candidacy for remediation, this study sought to characterize the algae's growth, protein expression, and nuclear integrity in fracking fluid. The algal population growth was negatively correlated with the percentage of fracking fluid present in the media. However, the population was sustained in as much as 1% hydraulic fracking fluid. While growth was inhibited, no general changes in overall protein expression levels were observed when comparing Coomassie stained SDS-PAGE gels of samples from media containing up to 10% hydraulic fracking fluid. *C. moniliferum*'s population stability and unaltered protein expression further supports its candidacy in remediating of water contaminated by hydraulic fracturing. Additionally, the nucleus and mitotic division of *C. moniliferum* were observed by novel incubation in 1%

toluene before DAPI staining. Nuclear integrity and mitotic fidelity are expected to be minimally affected by the presence mutagenic fracking fluid. Furthermore, the cytosolic Ba accumulation rate will be determined by flame atomic absorption spectroscopy (FAAS), while Ba binding to surface proteins will be characterized by Fourier transformed infrared spectroscopy (FTIR). While known to be incorporated as vacuolar statoliths, Ba's interaction with surface proteins and entrance into the algae cell is poorly characterized. It is expected that FTIR will reveal Ba interactions with the algae's sulfur rich surface proteins. The current and expected results support the use of *C. moniliferum* in the phycoremediation of water contaminated by hydraulic fracturing.

P679

Board Number: B932

Nandrolone decanoate and physical resistance training alter hormone levels and modulate AR, ER α and β expression in the ventral prostate of adult and aged rats.

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Nandrolone decanoate (ND) is a well known anabolic androgenic steroid, and its indiscriminate use leads to subclinical alterations in the hypothalamic-pituitary-gonadal axis, which can impair the functioning of androgen-dependent organs. This study was proposed to evaluate the effects of ND, associated or not with physical resistance training, on the morphophysiology of ventral prostate in adult and aged rats. Sprague-Dawley rats were divided into eight experimental groups (n=7): sedentary or trained and supplemented or not with ND. The groups were treated during adulthood for eight weeks. During this period the experimental groups were allowed to physical training with jump sessions in water (the animals conducted four sets of 10 jumps each, three times per week) and/or received the ND twice a week (5 mg / kg). Animals were euthanized after 48 hours post-treatment cessation and blood and tissue samples were collected for further analysis. The aged rats were subjected to the same experimental protocol during the adulthood, remaining untreated until reaching 300 days of age, when they were euthanized. The ventral prostate was processed for molecular and morphometrical analyzes to the androgen (AR) and estrogens (ER α and ER β) receptors, 5-alpha reductase 2 (5 α -R2), aromatase (cytochrome P450), proliferative nuclear cell antigen (PCNA) and protein pro-apoptotic 4 (PAR4). Testosterone levels were reduced in both adult and aged animals regardless of treatments, and estradiol increased only in the ND-treated groups. The expression of 5 α -R2 and aromatase was increased in exercised adult animals. Conversely, in aged groups was observed a reduction of 5 α -R2 in those exercised without ND, whereas aromatase expression was decreased in all treatments. The ER α and β increased in prostate of adult animals that received ND, and only ER β was reduced with age and treatment. AR expression was decreased in aged trained groups. The height of the prostate epithelium was reduced in all experimental adult groups compared to control group, coinciding with the increase in

PAR4 levels. Therefore, not only the ND, but also the modality of physical activity alter the hormone levels and differentially modulate the expression of AR and ERs. Overall, ND associated or not to physical resistance training during post-puberty stage is able to interfere with the morphophysiology of the prostate in adulthood and throughout the ageing process. Financial Support: FAPESP 2013/00649-7, 2013/07203-4.

P680

Board Number: B933

Copper Sulfide Nanoparticles: Potential Agents in Photothermal Therapy.

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The swiftly developing research field of nanomedicine has great potential in fighting against the complex disease of cancer. Photothermal therapy (PTT) is a minimally invasive therapy in which light energy is converted into heat to kill cancer cells. Nanoparticles, like gold, strongly absorb light and convert photon energy into heat quickly and efficiently, thereby making them superior agents for PTT. This gold nanoparticle-assisted PTT has shown great popularity and success in recent years. However, gold is expensive and the long term effects of this element in the body are not known. Copper Sulfide (CuS) is less expensive and its individual components are used by the body, even though in minute amounts. Therefore, the objective of this project was to determine if CuS can be used as a potential PTT agent in cancer cells. We hypothesized that CuS will be effective in killing cancer cells when used as a PTT agent. It has been reported that folate receptors are up-regulated in cancer cells. Therefore to test our hypothesis, we treated two cancer cell lines, A549 (lung cancer cell line, folate receptor negative) and HeLa (cervical cell line, folate receptor positive), with 0 or 100ng/mL of CuS, Folate and CuS + Folate for 24 hours. After 24 hours the cells were subjected to laser light at a wavelength of 900nm for 60 seconds. An MTT assay was then performed to determine the viability of cells. Preliminary results indicate that both cell lines treated with CuS and exposed to infrared light had decreased viability. However, HeLa cells that were treated with CuS + Folate had the greatest decrease in cell viability of all treatment groups. Therefore, CuS may be considered as a potential PTT agent.

P681**Board Number: B934****EU-OPENSREEN: Chemical tool compounds for cell biologists.**B. Stechmann¹, R. Frank¹;¹EU-OPENSREEN / FMP Leibniz-Institute for Molecular Pharmacology, Berlin, Germany

Small molecules that can be applied as chemical 'tool' compounds (or 'probes') have become indispensable in basic research for the elucidation of fundamental biological mechanisms. They act directly with the protein-of-interest and often allow for the interrogation of biological processes that cannot be properly studied with traditional genetic or RNA interference approaches.

EU-OPENSREEN (www.eu-openscreen.eu) is the largest emerging academic chemical biology research infrastructure initiative in Europe with the aim to collaboratively develop novel research tool compounds with independent cell biologists. As a joint effort of national networks in 16 European countries, EU-OPENSREEN offers access to high-throughput screening platforms, chemistry services and a large compound collection. It welcomes cell biologists who have a robust and suitable biological assay and are interested in collaboratively developing chemical tool compounds to validate their targets-of-interest. Selected assays are screened against a collection of 100,000-200,000 compounds, incl. confirmatory and counter screening, IC/EC50 determination, SAR (structure-activity relationships) and QC of confirmed hit compounds.

EU-OPENSREEN will start operations in late 2015, but it can already look back on a growing number of transnational activities: joint screening projects, exchange of local compound libraries, development of new design principles for its compound collection; exchange of experimental data through its pilot database etc.

Tissue Development and Morphogenesis 1**P682****Board Number: B936****Bone mineral and skeletal muscle formation induced by different gravitational exercise.**T. Sato¹, T. Watanabe², S. Igawa³;¹Department of Human Environmental Sciences, Jissen Women's University, Tokyo, Japan, ²Human Health Science, Hachinohe Gakuin University, Hachinohe, Japan, ³Dep. Health Science, Nippon Sport Science University, Yokohama, Japan

The constant influence of weightlessness during space flight leads to modifications of several physiological processes. Degradation of physical performance of astronauts after long-duration space missions is one of the topical but still unresolved problems of space medicine. The phenomenon is

commonly interpreted as a consequence of deconditioning of not only antigravity muscle but also bone structure because of long absence or reduction of mechanic and gravitational loads. It is well known that osteoporosis and muscle atrophy are affected by aging and insufficiency gravity stimulus as the human walking. The present study investigate the influence of such gravitational exercise as drop jump compare to swimming exercise in improving of bone minerals and muscle mass. The study was carried out with 21 Wister rats divided into three groups each 7 rats, i.e. gravitational exercise(drop jump), low gravitational exercise(swimming) and control. The experiments were performed after basically 5weeks breeding. During experiments periods for 8 weeks all animals were given standard laboratory diet and water available freely per day. It was increased gravitational effects on drop jump from 25cm(1 week), 30cm(2 week), 35cm(3 week) to 40cm(4-8 weeks) as gravitational training. In swimming group as low gravitational exercise were performed swimming 1 hour per day in 5 times per week by automatically pumping wave. The present study was carried out in accordance with the Japanese law, which allows experiments on laboratory animals in Nippon Sport Science University accordance to the Principles of laboratory animal care. After each one week exercise, hole femoris bone was isolated, bone density diagnosis both bone mineral content(BMC,g) and bone mineral density(BMD, g/cm^2). One-way ANOVA was used for statistical analysis of data between difference group, and P

P683

Board Number: B937

Deconstruction of a bio-mechanical ratchet during tissue morphogenesis.

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Myosin II motors generate forces that power cell shape changes during tissue morphogenesis. Such actomyosin networks function like a mechanical ratchet where phases of pulsed contraction and cell deformation alternate with phases of stabilization. *Drosophila* ectoderm extension exemplifies this differential behavior whereby pulses of acto-myosin flow towards junctions aligned in dorso-ventral axis leading to their shrinkage in order to facilitate cell intercalation(1). A planar polarized pool of Myosin II enriched at these junctions stabilizes the deformation resulting in an irreversible process(2). The mechanisms for regulation of these networks remain unknown. We investigated the role of dynamic phosphorylation of Myosin II regulatory light chain (RLC) by the Rho1-ROCK pathway. We find that spatial control over RLC phospho-cycles by Rok and Myosin II phosphatase mediates planar-polarized accumulation of Myosin II through regulation of its recruitment and dissociation. Investigating Myosin II temporal dynamics, we report that Myosin II pulses involve *de novo* recruitment of mini-filaments concomitant with their contraction, followed by disassembly. Interestingly, Myosin II pulses with its regulators Rho1, ROCK and Myosin II phosphatase. We refute a model where Myosin II pulsatility is enslaved by the upstream biochemical pacemaker Rho1. Instead, we propose a bio-mechanical feedback model postulating emergence of pulses through self-organization of its components. We find that pulsatility is set by the balance of recruitment of Myosin II by advection- mediated concentration of upstream regulators, and a delayed phosphatase-dependent depletion of Myosin II.

1. Rauzi, M., Lenne, P.F. & Lecuit, T. Planar polarized actomyosin contractile flows control epithelial junction remodelling. *Nature* 468, 1110-1114 (2010).
2. Bertet, C., Sulak, L. & Lecuit, T. Myosin-dependent junction remodelling controls planar cell intercalation and axis elongation. *Nature* 429, 667-671 (2004).

P684

Board Number: B938

PROBING COLLECTIVE MIGRATION OF A COMPLEX MULTI-CELLULAR EMBRYONIC TISSUE THROUGH NOVEL 3D BIOETCHING.

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Embryonic development consists of a complex series of cell signaling, cell migration and cell differentiation processes that are coordinated during morphogenesis. Collective cell sheet migration is an important process that sculpts the shape of an organism and its internal tissues during early development. Studies of these collective cell movements have focused on the behavior of epithelial monolayers of cultured cells such as Madin Darby canine kidney MDCK cells. By contrast, most embryonic development and tissue self-assembly requires the integration of cell movements within multiple cell layers composed of different cell types. Although the role of cell mechanics in tissue self-assembly has been demonstrated, little is known about the mechanical response of the multi-layer tissues to environmental cues. One of the reasons for this knowledge gap is the lack of the technologies to analyze the individual responses of epithelial and mesenchymal cell sheets in a multi-cell layer tissues. To investigate the processes that guide collective movements of multiple cell layers our group has focused on developing a novel microfluidic technique capable of producing complex patterns of multicellular structures. We call this technique "3D tissue-etching" by analogy with silicon micromachining techniques used to fabricate 3D microelectromechanical structures (MEMS) by successive steps to remove material from a monolithic solid. We use tissue etching to shape a complex multi-layered embryonic tissue and explore the dynamic collective responses of epithelial and mesenchymal cells in a single tissue. We use a custom-designed microfluidic control system to deliver a range of tissue etching reagents (detergents, chelators, proteases, etc.) over tissues microsurgically isolated from embryos of the African Claw-toed frog, *Xenopus laevis*. Using etching we produce free-edges of epithelial cells over mesenchymal cells and free-edges of mesenchymal cells. This allows us to study multi-layer coordination of epithelial and mesenchymal cell layers, as well as acute mechanical and behavioral responses of intact epithelial and mesenchymal cell sheets to the removal of neighboring or overlying tissues. The ability to control the forms of multicellular tissues will have high impact in tissue engineering and regeneration applications in bioengineering and medicine as well as in the synthesis of highly complex 3D integrated multicellular biosystems.

P685**Board Number: B939****RNA polymerase I transcription and craniofacial dysmorphology.**

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The transcription of rRNA by RNA polymerase I is the first step in making ribosomes in the nucleolus of eukaryotic cells. Transcription and the subsequent processing of the pre-rRNA requires hundreds of protein factors. Treacher-Collins Syndrome is an autosomal dominant craniofacial dysmorphology syndrome characterized by underdeveloped lower jaw and cheekbones, downward slanting eyes, conductive hearing loss, and malformed or absent ears. So far, mutation of 3 genes all involved in RNA polymerase I transcription have been found causative of Treacher-Collins Syndrome (TCOF1, POLR1C, or POLR1D). The latter 2 are also shared with RNA polymerase III. Thus, Treacher-Collins Syndrome is a ribosomopathy.

To further investigate a role for RNA polymerase I transcription factors in craniofacial dysmorphology, we have probed the role of two t-UTPs. t-UTPS, first characterized as essential proteins in the yeast, *S. cerevisiae*, are required for both RNA polymerase I transcription and pre-rRNA processing. We have explored whether intact UTP5 (WDR43) and NOL11 are required for normal development of the face in zebrafish and frog models, respectively. We have found that they are, and that disruption of their function leads to cranial neural crest apoptosis. The craniofacial defects can be rescued by inhibition of p53 function in both cases, demonstrating a conserved nucleolar stress response among vertebrates. Our results suggest the possibility of mutation of other nucleolar proteins in disorders of human craniofacial development.

P686**Board Number: B940****Small peptides induce selective ubiquitination to activate the transcription factor Shavenbaby by proteasome protein processing.**J. Zanet¹, E. Benrabah¹, T. Li², A. Pelissier-Monier¹, B. Ronsin¹, H. Bellen², F. Payre¹, S. Plaza¹;¹CNRS, Center of Developmental Biology, Toulouse, France, ²Neurological Research institute, Baylor College of Medicine, Houston, TX

Recent studies based on bio-informatics and ribosome profiling experiments revealed that a wide variety of RNAs contain small Open-Reading-Frames (smORFs) in yeast, invertebrates and mammals. However, the existence and the putative mode of action of smORF-encoded peptides remain elusive in the absence of direct evidence. The *Drosophila* *polished rice/tarsaleless* (*pri/tal*) gene encodes smORF peptides that control developmental processes. We previously showed that Pri peptides switch the transcriptional activity of Shavenbaby (Svb), a transcription factor required for epidermal differentiation. Here we demonstrate that Pri peptides induce a proteasome-mediated protein processing of Svb. Genome-wide RNAi screening identified the E3 ubiquitin ligase that targets, in a pri-dependent manner, Svb to the proteasome, which limited degradation ensures processing. As shown *in vitro* using synthetic peptides, we find that Pri peptides promote the binding of the E3 ligase to the Svb Nterminus. This in turn triggers ubiquitination of the Svb N-term degron and proteasome processing to release the activated form of the Svb transcription factor. Genetic ablation further demonstrates that this E3 ligase is essential for Svb processing *in vivo* and therefore for epidermal differentiation. These mechanistic insights into the action of Pri smORF peptides show how such small compounds can control proteasome action and, thereby, the execution of developmental programs.

P687**Board Number: B941****Annealing: the changing role of junctional actomyosin in epithelial cell packing during tissue development.**S. Curran¹, C. Strandkvist¹, B. Baum²;¹University College London, MRC Laboratory for Molecular Cell Biology, London, England, ²Laboratory for Molecular Cell Biology, University College London, London, United Kingdom

Epithelial morphogenesis is driven by actomyosin dependant changes in cell shape and neighbour exchange. Previous studies have focused on the polarised local movement of myosin during rapid morphogenetic processes, such as *Drosophila* germ band elongation. Less well understood is the role that the actomyosin cytoskeleton plays in regulating junctional rearrangements that occur in epithelia under conditions of homeostatic growth. As a model to investigate this process, we use the *Drosophila* *notum*. In this mono-layered epithelium, cell intercalation occurs without associated changes in tissue shape.

Here we show that actomyosin is not required to drive local cell rearrangements in this system. Conversely, when junctional levels of myo-II are increased through *sds22* RNAi, a negative regulator of myosin and moesin phosphorylation, junction length fluctuations and neighbour exchange events are inhibited. Interestingly, a similar change is observed during development as myosin progressively accumulates at junctions. Increased junctional myosin correlates with reduced junction length fluctuations and reduced neighbour exchange. This shift in actomyosin organisation appears to function like annealing to ensure that cell packing gradually orders over developmental time.

During rapid morphogenesis polarised junctional actomyosin provides the forces required for irreversible neighbour exchange and changes in global tissue shape. Based upon our data we propose that junctional sliding due to myosin-independent fluctuations contributes to cell packing and tissue refinement in epithelia under resting conditions.

P688

Board Number: B942

Apical mitotic position in pseudostratified epithelia is independent of the centrosome but facilitates cell reintegration following division.

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Pseudostratified epithelia (PSE) are proliferating tissues prevalent during animal development. These tissues feature a single layer of elongated cells whose nuclei adopt various positions along the apico-basal axis. Strikingly, regardless of their interphase position, all nuclei migrate towards the apical surface before mitosis. These nuclear movements result in apical localization of all divisions that canonically ensue with perpendicular cleavage planes. Following division nuclei are displaced from the apical surface and acquire more basal positions. This bidirectional movement of nuclei has been known as interkinetic nuclear migration (IKNM). To date, substantial insights into mechanics of this phenomenon have been gathered. However, the purpose of this nuclear migration and the resulting apical divisions in PSE remain elusive.

Notably, in PSE centrosomes localize apically. This means that in the interphase the centrosome and the respective nucleus remain at a distance. As centrosomes serve as spindle organizing centers, the centrosome and the nucleus associate before mitosis. It has therefore been proposed that apical nuclear migration in PSE ensures that nuclei and centrosomes meet for mitosis. We aimed to test this hypothesis. Surprisingly, we uncovered that apical IKNM occurs independently of the centrosome. We show that apical migration of nuclei takes place in cases when the centrosome and the nucleus meet non-apically. In this scenario, cells can enter mitosis at non-apical position. However, despite non-apical mitotic entry, the actomyosin-based machinery driving apical migration is still activated, resulting in the movement of the cell soma towards the apical surface. We additionally demonstrate that apical nuclear migration takes place even in cases when the integrity of the apical centrosome is perturbed. Collectively, this implies that apical IKNM is a highly robust phenomenon.

Next, we aimed to shed light on the cause of this remarkable reproducibility of apical nuclear migration and apical divisions. To achieve this we introduced non-apical divisions within the intact PSE. We observe that such ectopic divisions impair reintegration of the daughter cells into the epithelial structure and lead to cell delamination from the tissue. These delaminated cells continue proliferating basally, forming clusters that interfere with tissue architecture and subsequent maturation. Interestingly, we also demonstrate that upon the perturbation of the perpendicular cleavage planes of apical divisions tissue integrity is not compromised. We therefore propose that positioning all dividing nuclei apically safeguards the integrity of the PSE by allowing the cells to reintegrate into the epithelium following division.

P689

Board Number: B943

O- linked N-acetylglucosamine transferase accumulates at *Mgea5* , β -N-acetylglucosaminidase gene loci in mouse trophoblast stem cells.

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The placenta, which expresses numerous physiological factors such as hormones and enzymes, plays an important role during fetus development. The sensing of the maternal and fetal metabolism in the placenta is needed to achieve gene regulation. UDP-GlcNAc, a donor substrate for O-GlcNAcylation, exists abundantly in cells and is produced by the hexosamine synthetic pathway, which interacts with several other pathways such as amino acid and fatty acid metabolism. O-GlcNAcylation has recently emerged as an administrative system of epigenetic regulation via the modification of nuclear proteins, including histones. The enzyme O-GlcNAc transferase (OGT) catalyzes the O-GlcNAcylation of various proteins, and the enzyme β -N-acetylglucosaminidase (O-GlcNAcase) removes the O-GlcNAc from proteins. OGT gene is located in the X chromosome, and the gene encoding O-GlcNAcase has been mapped to chromosome 19, as *Mgea5* in the mouse genome. Trophoblast stem cells (TSCs) exhibit multipotency and differentiate into all of placental cells as spongiotrophoblasts and trophoblast giant cells. We noticed that *Ogt* expression is much higher in undifferentiated TSCs than in the differentiated ones. The protein expression level was confirmed with western blotting and immunostaining. To investigate the role of O-GlcNAcylation in the placenta, we determined the OGT target loci in undifferentiated and differentiated TSCs using chromatin immunoprecipitation sequencing (ChIP-seq) analysis. The genomic target loci were found to be much less abundant in undifferentiated TSCs than in the differentiated ones. More than 10,000 OGT target loci were identified in the differentiated TSCs, in contrast to less than 1,000 in the undifferentiated ones. This result suggests that OGT localizes to the nucleosome regions during differentiation. Alternatively, in the undifferentiated TSCs, almost all of the OGT may exist in the extra-nucleosome area. Both undifferentiated and differentiated TSCs showed accumulation of OGT around the transcription start sites and gene body. Intriguingly, the accumulation of OGT was detected by ChIP-PCR at the *Mgea5* locus in the differentiated TSCs, but not in the TSCs or in the mouse embryonic stem cells. Thus, *Mgea5* was one of the OGT targets that is present only in

differentiated TSCs. *Ogt* overexpression in differentiated TSCs led to increase in the *Mgea5* expression, which suggests that *Ogt* expression facilitates *Mgea5* expression. This phenomenon might be necessary for maintaining the O-GlcNAcylation levels in the trophoblast cells. In conclusion, OGT change the target loci dramatically before and after differentiation in TSCs. In addition, there is an intimate relationship between *Ogt* and *Mgea5* for genome-wide regulation of O-GlcNAcylation.

P690

Board Number: B944

A critical role of an exon junction complex (EJC) factor, RBM8a, in regulation of embryonic cortical progenitors.

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Nonsense-mediated mRNA decay (NMD) is a well-known RNA surveillance mechanism that ensures the speedy degradation of mRNAs carrying premature termination codons (PTCs). This mechanism relies on several key exon junction complex (EJC) factors to distinguish PTC from the normal stop codon. But how this mechanism modulates the embryonic brain development is largely unknown. Interestingly, mutation in *Magoh*, an important EJC factor, leads to defective neural stem cell division and microcephaly in mice. Human mutations in *Upf3b* have been reported to cause X-linked mental retardation and autism. This indicates that NMD may represent a novel pathway that regulates brain development and behaviors. Recently, we have identified that another key EJC factor, RBM8a, regulates anxiety behaviors in mice, further supporting the importance of EJC factors in brain functions. In this study, we demonstrated that RBM8a plays a key role in neural progenitor proliferation and differentiation. First, RBM8a is highly expressed in the subventricular zone of early embryonic cortex, suggesting that RBM8a may play a role in regulating neural progenitor cells (NPCs). To test this hypothesis, we used in utero electroporation to overexpress or knock down RBM8a in mouse brain at E14. RBM8a stimulates embryonic neural progenitor proliferation and suppresses neuronal differentiation, indicating that RBM8a positively regulates NPC proliferation. Conversely, knockdown of RBM8a in the neocortex reduces NPC proliferation and promote premature neuronal differentiation. Moreover, overexpression of RBM8a suppresses cell cycle exit and keeps cortical NPCs in proliferative state. Severe morphological defects were observed in immature neurons. Consistently, *Nes-cre*; RBM8a^{fl/+} mice show severe developmental defects including microcephaly and postnatal lethality. When RBM8a is overexpressed in the dentate gyrus of adult brain using stereotactic viral injection, mice showed altered anxiety and depressive-like behaviors. To uncover the underlying mechanisms, genome-wide RNA-immunoprecipitation-RNAseq identifies potential substrates of RBM8a in the brain, which have been implicated in neurogenesis and plasticity. Interestingly, autism- and schizophrenia-risk genes are highly representative in RBM8a-associate transcripts. Taken together, we identify a novel role of RBM8a in regulation of neurodevelopment and behaviors. Our studies provide a deeper insight on

causes of mental illnesses and will facilitate the development of new therapeutic strategies for neurodevelopmental illnesses.

P691

Board Number: B945

the Endocytic Recycling Regulator EHD1 regulates Ciliary Morphogenesis and Signaling during Neural Tube Closure.

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EHD1 is a member of the highly conserved dynamin-like C-terminal Eps15 homology domain-containing (EHD) protein family that function as essential components of endocytic recycling in cell systems. To define its biological functions in mammals, we have engineered *Ehd1* deletion in mice. In a predominantly B6 background, *Ehd1* deletion resulted in mid-gestation embryonic lethality. We show that EHD1 is expressed early during embryonic development, and that *Ehd1*-null embryos exhibit defective neural tube closure, axial turning and patterning of the neural tube. Importantly, *Ehd1*-null embryos exhibit stubby primary cilia architecture in the developing neural tube and this phenotype is recapitulated in *Ehd1*-null mouse embryonic fibroblasts together with a decrease in the number of primary cilia. As primary cilia are hubs for Hedgehog (Hh) signaling in vertebrates, we analyzed downstream effectors of Hh signaling and found that *Ehd1*-null embryos exhibit features of increased Sonic Hh (Shh) signaling with a severe down-regulation in Gli3 repressor expression. The *Ehd1*-null embryonic neural tubes showed reduced expression of markers for dorsal cell identities (Pax6 & Pax7) and severely restricted dorsal domains, with a moderate expansion of markers for ventral identities (Foxa2 and Nkx6.1). Based on our observations, we suggest that EHD1-regulated membrane traffic is a critical determinant of primary cilia morphogenesis and Shh signaling. Further studies of the model we have developed should enhance our understanding of primary cilia morphogenesis and may provide avenues to better understand the pathogenesis of neural tube defects, one of the most common birth defects occurring in approximately one in 1,000 live births in the United States.

P692**Board Number: B946****Investigation of Caprin2 function in lens fiber cell homeostasis.**S. Dash¹, C.A. Dang², D.C. Beebe³, S.A. Lachke^{2,4};¹UDel, Newark, DE, ²Biological Sciences, University of Delaware, Newark, DE, ³Department of Ophthalmology and Visual Sciences, Washington University School of Medicine, St. Louis, MO, ⁴Center for Bioinformatics and Computational Biology, University of Delaware, Newark, DE

The eye disease cataract is the leading cause of blindness worldwide and is caused by the loss of lens transparency. In order to prevent or delay cataract, it is essential to understand the molecular mechanisms that contribute to the formation and maintenance of lens transparency. We recently developed a bioinformatics tool iSyTE (integrated Systems Tool for Eye gene discovery) that identifies genes highly enriched in mouse lens development and uses this information to effectively predict candidates whose deficiency associates with mammalian cataracts. Using iSyTE we recently identified two post-transcriptional regulatory proteins (Tdrd7 and Celf1) that are components of cytoplasmic RNA granules, and are essential for vertebrate lens development. This is demonstrated by the severe lens defects including cataracts that are caused by their deficiency in multiple animal models, in turn suggesting a conserved function for RNA granule component proteins in lens development across diverse species. Here we investigate the function of a third conserved RNA granule component protein Caprin2 that is newly identified by iSyTE to be associated with lens biology. Caprin2 protein has multiple conserved domains including the coiled-coil and RGG domains as well as the C1q domain, which is found in TNF super-family of proteins to facilitate protein-protein interactions. Interestingly, Caprin2 expression has been recently shown to be responsive to induction by FGF8 in chicken lens fiber cells, supporting a potential role in the lens. As predicted by iSyTE, we confirmed by performing qRT-PCR, in situ hybridization, western blotting and immunostaining that Caprin2 mRNA and protein exhibits highly lens-enriched expression in mouse embryonic and postnatal lens. To functionally characterize this protein, we crossed mice obtained from EUCOMM that carried Caprin2 conditional mutant alleles (Caprin2^{tm2a(EUCOMM)}Wtsi) with an established lens Cre deleter line Pax6GFPCre to generate lens-specific Caprin2 conditional knockout (cKO) mutants. Phenotypic characterization has revealed that Caprin2 cKO mutants exhibit, albeit with incomplete penetrance, lens and eye defects, including a developmental defect called Peter's Anomaly. Scanning electron microscopy demonstrates that Caprin2 cKO mutants have a mild but consistent abnormality in lens fiber cells that contribute to the bulk of the lens tissue. Interestingly, Caprin2 has been associated with terminal differentiation of erythroblasts from a highly proliferative state into cells devoid of nuclei, similar to the transition of lens epithelial cells into terminally differentiated fiber cells that undergo nuclear degradation for lens transparency. In sum, our data has revealed a new function for Caprin2 in mammalian lens fiber cell.

P693**Board Number: B947****The role of N-cadherin in lens fiber cell elongation and lens morphogenesis during development.**S. Rajakaruna¹, C.M. Logan², G.L. Radice³, S. Menko⁴;¹Thomas Jefferson University, Philadelphia, PA, ²Anatomy, Thomas Jefferson University, Philadelphia, PA, ³Thomas Jefferson Univ, Philadelphia, PA, ⁴Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, PA

The process of lens fiber cell elongation is a central feature of lens morphogenesis. We investigate the role of N-cadherin junctions in this process in vivo. Here, we show how molecular interactions between N-cadherin and cytoskeleton regulate lens morphogenesis. Our study focus on investigating how N-cadherin function is integrated with microfilaments (MFs) and microtubules (MTs) to promote fiber cell elongation, and to examine the molecular regulators of N-cadherin/cytoskeletal interactions in fiber cell elongation and morphogenesis. To evaluate the impact of N-cadherin we generated a lens fiber cell specific NcadcKO with MLR10 Cre mice. In MLR10 NcadcKO lens at early E13.5 showed a change in directionality in MFs and MTs, while at E18.5 there was a dramatic change in MFs/MTs directionality and deregulation, and subsequent epithelial-fiber interface (EFI) separation in the lens. Proteins that link N-cadherin complexes to cytoskeleton such as β -catenin, and acetylated-tubulin identifying the stable microtubule populations were also suppressed in MLR10 NcadcKO lenses. Myosin II activation required for MF based cell motility was also deregulated as there was an increased expression of phospho Myosin (p-Myosin) in MLR10 NcadcKO lenses. Our preliminary data strongly implicates the importance of N-cadherin as the key focal point for lens fiber cell elongation and morphogenesis in lens cell development, and may serve as crucial knowledge required for designing effective strategies in regenerative medicine.

P694**Board Number: B948****A conserved RNA binding protein Celf1 is essential for vertebrate lens development and fiber cell differentiation.**A. Siddam¹, C. Gautier-Courteille², V. Legagneux², A. Kakrana³, A. Méreau², C.A. Dang¹, J. Viet², D. Scheiblin¹, L. Perez-Campos⁴, D.C. Beebe⁵, J. Gross⁴, L. Paillard², S.A. Lachke^{1,3};¹Biological Sciences, University of Delaware, Newark, DE, ²Institut de Génétique et Développement de Rennes, Université de Rennes, Rennes, France, ³Center for Bioinformatics and Computational Biology, University of Delaware, Newark, DE, ⁴Department of Molecular Biosciences, University of Texas, Austin, TX, ⁵Department of Ophthalmology and Visual Sciences, Washington University School of Medicine, St. Louis, MO

The ocular lens is a transparent tissue made of epithelial and fiber cells that focuses light on the retina to enable high-resolution vision. Loss of lens transparency results in cataract - the leading cause of blindness affecting 40 million worldwide. While the significance of signaling molecules and transcription factors in lens development, homeostasis, and cataractogenesis is well characterized, that of post-transcriptional regulators is unclear. Post-transcriptional regulation - controlled by RNA binding proteins (RBPs) among other molecules and defined as regulation of RNA transcript processing, localization, stability, degradation, and translation - is essential for cell function. Yet, while the human genome encodes 1500 RBPs, less than 20 are associated with development or disease. To identify new regulators associated with lens differentiation and cataract, we developed a novel systems tool iSyTE (integrated Systems Tool for Eye gene discovery) and reported that deficiency of an RNA granule (RG) protein Tdrd7 results in juvenile cataracts in human and mouse (Lachke 2011 Science 331:1571). Here, we report a new iSyTE-identified RG-RBP Celf1 (Cugbp1) that functions in the post-transcriptional control of gene expression in vertebrate lens fiber cell differentiation. Celf1 has three RNA Recognition Motifs that bind to target RNAs to control mRNA decay, pre-mRNA alternative splicing or translation. We find that Celf1 exhibits a highly fiber cell-enriched expression pattern that is conserved in fish, frog and mouse. To investigate its function in fiber cells, we generated and characterized both germline and lens-specific Celf1 deletion mouse mutants. We find that Celf1^{-/-} mice develop fiber cell defects during embryogenesis that result in severe cataracts at birth. Significantly, Celf1 knockdown in Zebrafish and Xenopus also causes lens defects. Moreover, Celf1 deficient Zebrafish and mouse mutants exhibit fiber cell nuclear degradation defects emphasizing its functional conservation across diverse vertebrate species. Interestingly, microarray-based expression profiling of Celf1^{-/-} mouse lens reveals down-regulation of Dnase2b, an enzyme necessary for nuclear degradation in fiber cell differentiation. Furthermore, Celf1 deficiency results in mis-regulation of the cyclin-dependent kinase inhibitors p21 and p27 and of the F-Actin cross-linking protein Actn2. Finally, we find that Celf1 directly binds to Dnase2b and p27 transcripts and regulates their stability or translation into protein, respectively. In sum, we describe a new function for Celf1-mediated control mechanisms that critically function in vertebrate lens development by regulating diverse cellular properties such as fiber cell differentiation, cell cycle control and cell cytoskeleton.

P695

Board Number: B949

Role of Dnmt1 in photoreceptor differentiation.

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DNA methylation is a major epigenetic mechanism that participates in retinogenesis during eye development, and is facilitated by DNA methyltransferases, which catalyze the transfer of a methyl group to cytosine during DNA replication. This process leads to gene silencing in the genome. Objective: This study focuses on the role of DNA methyltransferase-1 (Dnmt1) in photoreceptor differentiation

during retinogenesis. DNMT1 (maintenance DNA methyltransferase), plays a significant role in replicating the parental DNA methylation patterns in the daughter DNA strands. Reducing Dnmt1 expression or/and introducing deletions into functionally important parts of the protein leads to reduction of DNA methylation. Mammalian retina is an excellent and simple model to study the impact of epigenetics (DNA methylation) on cell fate determination during Central Nervous System (CNS) development. Only six different neuronal cell types, one glial cell type and one pigmented cell type are produced during retinogenesis. Rod and cone photoreceptors play a major role in phototransduction and originate from bipotential Crx[+] rod-cone progenitors. Methods: We chose the Nrl knockout (Nrl -/-) mouse model where the loss of neural retina leucine zipper transcription factor leads to conversion of all rods into cones. This model of cone-only retina is a valuable model of human macula. This model, in combination with the conditional Dnmt1 knockout model, generated in our previous studies, is used here to delineate the impact of DNA demethylation on the conversion of rods into cones. We knocked out Dnmt1 in mouse retina using Rx-cre transgene in an Nrl -/- background and determined the impact of partial DNA demethylation on the conversion of rod photoreceptors into cones. We evaluated changes in gene expression, histology and started comparing changes in retinal function (electroretinogram) in Dnmt1 flox/flox, Rx-Cre, Nrl -/- “mutant” mice and “control” Nrl -/- animals. Results: First, we validated this model at the mRNA level and found no expression of Nrl and Rhodopsin in both “control” and “mutant” retinas. Next, we found the upregulation of genes such as Chx-10 and Otx2 (expressed at earlier stages of photoreceptor development) in mutants, while the S-opsin and M-opsin genes were downregulated in mutants. Further, we found some changes in retinal histology in mutant mice such as decreased number of rosettes, typical for Nrl -/- retina. Conclusions: Reduction of DNA demethylation in developing retina may impact cone photoreceptor development in this cone-only model. These data highlight the need to further investigate the role of Dnmt1 in photoreceptor development.

P696

Board Number: B1000

TRPV4 channel is necessary for appropriated establishment of the tight junctions in corneal epithelium and regulates its barrier function in combination with EGF.

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We are interested in identifying ionic channels that regulate the proliferation and/or differentiation of epithelial cells. We study the spontaneously immortalized cell line RCE1-5T5, from rabbit corneal epithelium, which reproduces in vitro early stages of differentiation of this tissue. After screening for ionic channels differentially expressed between proliferative and differentiated RCE1-5T5 cultures, we observed that TRPV4 channel in differentiated culture is localized in the apical membrane of the more external cells layer in the stratified epithelium while in proliferative, cells it is expressed homogeneously in the cell. In spite of this, its mRNA levels remains constant and protein channel decreases 3.5 folds

when cells differentiate. TRPV4 is a cationic channel, activated by hypotonic cell swelling, heat, endogenous agonist like arachidonic acid and endocannabinoids and synthetic specific agonists 4 α -PDD and GSK1016790A (GSK101). It has been reported that TRPV4 mediates the regulatory volume decrease behavior in human corneal epithelial cells. TRPV4 also regulates the epithelial barrier function of tight junctions (TJ) in skin keratinocytes, mammalian gland cells and lung endothelium. With these antecedents we first demonstrate by calcium imaging that TRPV4 is functional in RCE1-5T5 cells and induces an increment of intracellular calcium in the presence of its specific activator GSK101 (100 nM) which is avoided by treatment with RN1734 (30 μ M), an specific blocker of TRPV4 or EGTA (2mM). We then analyzed the role of TRPV4 on TJ recording the Transepithelial Resistance (TER) of RCE1-5T5 cultures grown in presence of GSK101 or RN1734. We found that TRPV4 is necessary for the establishment of TJ. Additionally in mature epithelia, activation of TRPV4 increases the TER value. This effect seems to be mediated by augmentation of claudin 1 and 4 in the cell-cell contact of the most apical cell layer. It has been reported that TRPV4 as heteromer with TRPP2 forms an EGF activated channel at the apical membrane of renal collecting duct cells. On the other hand, EGF modulates TER in RCE1-5T5 cells. Therefore we evaluated the possible participation of TRPV4 in the regulation of TER in RCE1-5T5 by EGF. We found that activation of TRPV4 can enhance the values of TER in absence of EGF while the blockade of TRPV4 avoids the normal increase of TER even in presence of EGF. In conclusion our results suggest that TRPV4 is necessary for the correct establishment of the barrier function of TJ in corneal epithelia. Additionally in the differentiated epithelium, activation of TRPV4 regulates the barrier function of TJ increasing the values of TER and it is also involved in the regulation of TJ by EGF.

P697

Board Number: B1001

Gene expression and the differentiation of gastric epithelial cells: can early weaning disturb the developmental program?.

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Background: Rat gastric epithelial cells differentiate during the third postnatal week and complete functional maturation is detected on suckling-weaning transition. From the five main cell populations, mucous and zymogenic cells depend on the expression of genes that regulate the activity of secretory apparatus. It is known that early weaning (EW) disrupts the gradual nutritional and behavioral transitions, but it also disturbs the differentiation program. Objective: In order to test how EW alters gastric epithelial cell differentiation and to check whether changes persist in young adults, we evaluated the expression of genes involved in the maturation of secretory apparatus in mucous (MC) and zymogenic cells (ZC), and studied the distribution of mucous neck cells. Methods and Results: Wistar rats were submitted to EW at 15d (protocol approved by Ethical Committee on Animal Use, ICBUSP) and gastric samples were collected at 18, 30 and 60d for qRT-PCR. Mist1 acts in the terminal differentiation of mucous neck cells (MNC) into ZC, and we found that EW immediately decreased mist1 expression (p

P698**Board Number: B1002****MODIFYING PROSTATE GLAND EPITHELIAL PHENOTYPES WITH FATTY ACIDS.**D.L. Ferrucci¹, H.F. Carvalho²;¹Structural and Functional Biology, State University of Campinas, Campinas/SP, Brazil, ²Structural and Functional Biology Department, State University of Campinas, Campinas SP, Brazil

Prostate cancer (PCa) is the second most common malignancy among men and benign prostate hyperplasia (BPH) is the fourth prevalent disease in men over the age of 50 years. Prostate growth (either PCa or BPH) is multifactorial and may be influenced by genetic, age, ethnicity, hormonal, exercise engagement and nutritional factors. Experimental data indicate that the amount of fatty acid in the diet is positively correlated with prostate gland disorders, and hence diets rich in fatty acids cause prostate enlargement. As a matter of fact fatty acid quality interferes with prostate growth, but this is poorly studied. We showed before that fatty acid quality affects cell proliferation with lard promoting and linseed oil having opposite effects. The objective of this study was to investigate the effects of fatty acid quality in normolipidic diets on the growth and gland architecture of the ventral prostate in C57/BL6 mice. Animals were divided into three groups fed with isocaloric normolipidic diets (7% fat) containing soybean oil, linseed oil and rendered pork fat (lard) for 10 and 32 weeks starting immediately after weaning. The results demonstrate that diets resulted in similar weight gain until the tenth week, but linseed oil resulted in leaner bodies, as measured as total body density. After puberty, the soybean oil diet resulted in higher body weight and ectopic fat pad, animals treated with lard and linseed oil presented similar weights gains and body composition. Lard and soybean oil based diets promoted increased prostate weight, while the linseed oil resulted in smaller prostates with an atrophic epithelium. Stereology demonstrated that lard and soybean oil based diets promoted prostate enlargement and altered tissue architecture, with increased epithelium and smooth muscle cell volumes. Interestingly, the group fed with saturated fatty acid content contributed to a decrease in lumen compartment. Increased androgen receptor content was determined by Western blots in the group fed with lard, with the opposite effect seen with linseed-based diets, which caused reduced androgen receptor content. The present results showed that lard results in a hyperplastic epithelium, while the linseed oil resulted in epithelial atrophy. It was remarkable that soybean oil caused animal and prostate growth. Continuing analysis will focus on the mechanism contributing to the prostate phenotype.

P699**Board Number: B1003*****MacF1* is Essential for Visceral Endoderm Development in Mouse Embryos.**K.M. Gonzalez¹, J. Rivera-Perez²;¹University of Massachusetts Medical School, Holden, MA, ²Cell and Developmental Biology, University of Massachusetts Medical School, Worcester, MA

Microtubule Actin Crosslinking Factor 1 (MacF1) a member of the spectraplakins family of proteins, binds actin filaments and microtubules in the cytoskeleton. *MacF1* is a positive regulator in the *Wnt* signaling pathway and its deficiency is lethal at early post-implantation stages in mouse embryos. In zebrafish, *MacF1* is essential for axial specification and its maternal deficit leads to the failure to establish the molecular asymmetry responsible for the formation of the anteroposterior axis. To understand the role of *MacF1* in murine axial specification and development we generated maternal and tissue specific mutations in mice. Our results show that *MacF1* acts downstream of *Wnt3* during gastrulation and that it is essential for the development of the visceral endoderm (VE). *MacF1*-VE mutant embryos show abnormal posterior VE cell structure and abnormal gastrulation compared to wild type littermates. Lack of maternal *MacF1*, however, does not affect embryonic development. These results suggest that *MacF1* has a fundamental role in post-implantation mouse embryos but unlike zebrafish, maternal *MacF1* does not appear to play a role in axial development in mouse embryos.

P700**Board Number: B1004****The role of iASPP in epithelial cell motility.**Z. Dedic¹, X. Lu^{1,2};¹Nuffield Department of Medicine, The University of Oxford, Oxford, United Kingdom, ²Nuffield Department of Clinical Medicine/ Ludwig Cancer Research, The University of Oxford, Oxford, United Kingdom

The majority of cancer-related mortality is due to cancer metastasis, a biological process that involves cell migration, proliferation and resistance to apoptosis. These processes also occur during morphological and physiological events including embryonic eyelid closure and wound healing. In addition, signalling pathways implicated in eyelid closure and wound healing are involved in metastasis. Examination of the regulation of molecular pathways involved in embryonic eyelid closure and wound healing could give us insight into the molecular mechanisms of metastasis. One of the main eyelid closure signalling pathways—the EGFR pathway—is deregulated in many cancers. The deregulation of EGFR has been associated with resistance to cancer therapies and worse prognosis for cancer patients. This led to generation of anti-EGFR cancer therapies, which yielded only moderate efficiency due to acquired cancer resistance to EGFR inhibiting treatments. p53 regulates EGFR. Loss of p53 and p53-induced cell cycle arrest and apoptosis is thought to contribute to acquired cancer resistance to EGFR treatments. iASPP binds and regulates p53-induced pro-apoptotic transcriptional activity. Lack of iASPP

in mouse models results in the eyelid-open at birth (EOB) phenotype, wavy hair and heart defects. These phenotypes are also observed in mice with deregulated EGFR signalling suggesting a potential role of iASPP in the regulation of EGFR signalling, which could be p53 dependent. Here we studied a potential link between iASPP and EGFR signalling by examining histological and molecular mechanisms by which loss of iASPP induces EOB phenotype. We show EOB phenotype in iASPP mutant mice is due to impaired epithelial sheet progression, which is not due to altered proliferation. Increased apoptosis is one of the contributing factors for this trait. In vitro analysis of migratory behaviour of keratinocytes showed faster and disorganized motility in iASPP deficient cells, implicating migration defect in impaired embryonic eyelid closure. Consistent with this, iASPP is highly expressed in the migratory cells of eyelid epithelia and wound closure, which depends on keratinocyte migration, is delayed in absence of iASPP in vivo. Increased expression of phosphorylated ERK, a downstream component of EGFR signalling, observed in iASPP deficient keratinocytes suggests that iASPP could regulate EGFR signalling. Future studies will aim to elucidate the mode of action of iASPP in EGFR pathway, and what effects this has on motility. Moreover, the mechanistic regulation of iASPP in the mediation of motility will be examined. It is hoped that this study, will extend our understanding of some of the mechanisms that influence cancer progression, and facilitate the development of new therapeutic strategies.

P701

Board Number: B1005

New mammary epithelial ducts form through coordinated polarization of the initiation of subcellular protrusions.

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Morphogenesis in the mammary gland occurs iteratively during embryonic, fetal, and postnatal development. During each stage of morphogenesis, the mammary epithelium undergoes characteristic changes in architecture, including stratification and loss of apico-basal polarity. Cells within elongating epithelial buds migrate collectively, which raises the question of how these adherent cells can accomplish directional migration. Single cell migration can be guided through control over the polarization of protrusive activity. We sought to understand whether a similar mechanism could explain the initiation and elongation of mammary epithelial ducts.

The mammary stroma is composed of numerous adipocytes that scatter light and make direct observation *in vivo* highly challenging. To overcome this barrier, we developed 3D organotypic culture techniques to study morphogenesis in real-time. Briefly, the mammary gland is isolated, enzymatically digested, and the resulting epithelial organoids are explanted into 3D extracellular matrix gels. Next, we image the cellular dynamics through mosaic fluorescent labeling of single-cells within these organoids during branching morphogenesis.

We are able to image the initiation of new epithelial buds, ductal elongation, and polarization to a mature simple epithelium. We observe active cell migration during each of these processes and

cytoplasmic labeling revealed extensive protrusive activity within the epithelial group. Cells initiating new buds move in concert and initiate protrusions preferentially in the direction of tissue growth. We observe a diversity of protrusive shapes and individual cells can exhibit one dominant protrusion or many fine protrusions. Cells distant from the elongating duct initiate protrusions in an undirected manner and their migration is uncoordinated with their neighbors. Cells contributing to elongating ducts initiate more protrusions, display a greater polarization of protrusive activity in the direction of elongation, have higher velocities, and migrate over longer distances, when compared to cells in the body of the organoid.

Taken together, our data suggest a model in which epithelial ducts initiate through the coordinated polarization of protrusive activity. We are now focused on understanding how external cues are mediated through receptor tyrosine kinase signaling to control the orientation of protrusive activity and the coordination of migratory behavior.

P702

Board Number: B1006

Polycystin-1 binds Par3/aPKC and controls Convergent Extension during Renal Tubular Morphogenesis.

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Several organs, including lungs and kidneys, are formed by epithelial tubes whose proper morphogenesis ensures correct function. This is best exemplified by the kidney, where defective establishment or maintenance of tubular diameter results in polycystic kidney disease (PKD), a common genetic disorder. Most PKD cases result from loss-of-function mutations in the PKD1 gene, encoding Polycystin-1 (PC-1), a large receptor of unknown function. Here we demonstrate that PC-1 plays an essential role in establishment of correct tubular diameter during nephron development. Counting the number of cells in the cross-section of DBA-positive tubules in wild-type or Pkd1 mutant kidneys at different developmental stages (from E13.5 to E16.5) revealed that, while the tubules in wild-types become progressively narrow, in Pkd1 mutants this property is impaired. No significant differences in proliferation or apoptosis were detected to explain this defect. Conversely, morphometrical analysis of the epithelium lining the tubules of Pkd1 mutants revealed a failure to achieve mediolateral cell orientation, essential for a convergent-extension(CE)-like process occurring during renal tubular morphogenesis. In line with this, cells lacking PC-1 fail to properly orient during cell migration. Using cells and tissues derived from a mouse line expressing HA-tagged endogenous PC-1, we found that PC-1 associates with Par3 favoring the assembly of a pro-polarizing Par3/aPKC complex. Furthermore, PC-1 and Par3 undergo a similar regulation of expression during renal development. GST pull-down assays revealed that the C-terminal tail of PC-1 directly associates with the first two PDZ domains of Par3. Finally, we found that PC-1 regulates a Par3/aPKC complex important for oriented cell migration and for

CE during tubular morphogenesis. Consistently, Par3 inactivation in the developing renal tubules results in defective number of cells per tubular cross-section, defective mediolateral cell orientation and renal cystogenesis. Our data define PC-1 as central to cell polarization and to epithelial tube morphogenesis and homeostasis.

P703

Board Number: B1007

Tuning Myosin Activity to Investigate How Myosin Drives Cell Shape Change.

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Cytoskeletal dynamics govern changes in cell shape that form the basis of tissue morphogenesis. Gastrulation is a critical developmental process in which a single sheet of cells folds inward. In many organisms, apical constriction drives cell shape change to produce tissue folding. This can occur through contraction of the actomyosin network at the apical surface of constricting cells. We would like to understand how apical junctions become coupled to the contracting actomyosin networks, eliciting constriction of the apical cortex of cells. Here, we asked whether coupling is promoted by myosin-generated tension in the apical cortex. Phosphorylation of myosin's regulatory light chains (rMLC) at conserved sites leads to heavy chain assembly, permitting actomyosin network contraction. In *C. elegans* embryos, the endodermal precursor cells Ea and Ep are the first cells to undergo internalization, at the 26-cell stage of development. Prior to the onset of gastrulation, myosin accumulates apically and becomes activated in these cells. To investigate whether coupling is promoted by myosin-generated tension in the apical cortex, we attempted to tune myosin activity up or down and determine if this can lead to earlier or later cell internalization. Overexpressing a nonphosphorylatable form of myosin regulatory light chain in E cells delayed cell internalization, consistent with multiple models in which myosin activity is required for cell internalization. We tried increasing myosin activity by expressing a phosphomimetic rMLC construct in E cells or by overexpressing a kinase required for apical myosin activation. Interestingly, both methods led to delays in E cell internalization. Taken together, these results suggest that increasing myosin activity may not be sufficient to cause premature coupling and E cell internalization. We are currently examining whether the phosphomimetic rMLC and kinase overexpression increase myosin activity and tension in the cell cortex as predicted.

P704**Board Number: B1008****A critical and previously unsuspected role for Doublecortin at the neuromuscular junction in mouse and human.**

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The microtubule-associated protein doublecortin (DCX) is primarily expressed in post-mitotic neurons during embryonic development of the nervous system. Human mutations cause type I (X-linked or XLIS) lissencephaly in hemizygous males and subcortical band heterotopia in females (SBH), developmental disorders associated with brain malformations and characterized at the cellular level by defects in neuronal migration which affect cortical lamination.

We found that, besides the well-established expression in migrating neurons in brain, doublecortin is also expressed in embryonic motor neurons and skeletal muscle in mice, raising the possibility of a role in synaptogenesis. Studies with whole-mount preparations of embryonic mouse diaphragm revealed that lack of doublecortin leads to abnormal presynaptic arborization and significantly increased incidence of axonal extensions beyond innervated acetylcholine receptor clusters in the developing neuromuscular junction (NMJ). Our data suggest the contribution of doublecortin to a stop/stabilizing signal at the synapse which would normally limit further axonal growth following establishment of synaptic contact with the postsynaptic element. Importantly, we also identified abnormal and denervated NMJs in a muscle biopsy from a 16-year old female patient with SBH, showing both profound presynaptic and postsynaptic morphological defects.

Overall, these results point to a critical role of doublecortin in the normal establishment of the presynaptic element and suggest a possible involvement of this protein in the maintenance of NMJs in humans.

P705**Board Number: B1009****An essential morphogenetic role for Integrins in regulating tissue level tensile force by modulation of cell mobility.**E. Lostchuck¹, S. Ellis¹, K.E. Goodwin¹, D. Gunne¹, T. Zulueta Coarasa², S. Wistorf¹, J.J. Feng³, R. Fernandez-Gonzalez^{4,5,6}, G. Tanentzapf¹;¹University of British Columbia, Vancouver, BC, ²Univ Toronto, Toronto, ON, ³Department of Mathematics, University of British Columbia, Vancouver, BC, ⁴Institute of Biomaterials and Biomedical Engineering, University of Toronto, Toronto, ON, ⁵Department of Cell and Systems Biology, University of Toronto, Toronto, ON, ⁶Developmental and Stem Cell Biology Program, The Hospital for Sick Children, Toronto, ON

Integrin-mediated Cell-ECM adhesion is essential for tissue morphogenesis. Integrins facilitate morphogenetic processes by supporting cell migration, stabilizing cellular architecture, and by regulating cell behavior. Here, we describe an additional way that integrins contribute to morphogenesis: they ensure that the biomechanical parameters that exist within a tissue promote the desired morphogenetic outcomes. Our work focuses on Dorsal closure (DC), an integrin-dependent morphogenetic process that occurs during fly embryogenesis. We have identified novel integrin-containing adhesive structures on the basal surface of the amnioserosa, an extra-embryonic epithelium that is essential for DC. Since these in vivo structures share many features with focal adhesions we termed them Focal Adhesion-Like Structures (FALS).

Using mutations that either increase or decrease integrin-based Cell-ECM adhesion, we show that integrins regulate the mobility of amnioserosa cells within the two-dimensional epithelial sheet. Furthermore, using mathematical modeling, quantitative image analysis, and in vivo laser ablation experiments we find a direct correlation between cell mobility and the amount of tension in the tissue. Specifically, mutations that exhibit increased cell mobility within the tissue result in less tension, while mutations that decrease cell mobility result in more tension within the tissue. Finally, we show that mutations that alter cell mobility and tension within the amnioserosa result in defective DC. We are able to corroborate this data using a mathematical model of DC, in which modulation of the friction factor recapitulates the phenotypes that we observe in our mutants. Overall, our data demonstrates that integrin-mediated cell-ECM adhesion determines cell mobility, and consequently, tension within a tissue and that this regulation has profound effects on tissue morphogenesis. We propose a model whereby integrin-mediated Cell-ECM adhesion is required to maintain tissue-level tension and other biomechanical parameters within an ideal range that promotes morphogenesis. Our results therefore uncover a fundamental and likely conserved mechanism for developmental regulation that links integrin-based adhesion, tissue biomechanics, and morphogenesis.

P706**Board Number: B1010****The role of Tcf4 and connective tissue fibroblasts in myogenesis.**S.J. Mathew¹, G. Kardon²;¹Regional Centre for Biotechnology, Gurgaon, India, ²Department of Human Genetics, University of Utah, Salt Lake City, UT

The vertebrate musculoskeletal system comprises the muscle, muscle connective tissue, tendon and bone. The muscle connective tissue includes the extracellular matrix and the connective tissue fibroblasts, which secrete the extracellular matrix. We discovered that the Wnt/beta-Catenin pathway transcription factor Tcf4 is strongly expressed in the connective tissue fibroblasts, and weakly by myofiber nuclei. We also generated the Tcf4GFP-Cre mice where Cre mediated reporter activity was detected in the connective tissue fibroblasts, allowing genetic manipulation of these fibroblasts. Our results indicate that Tcf4 plays dual roles in myogenesis: first, an intrinsic role wherein Tcf4 expressed by myogenic cells regulates both slow and fast muscle fiber type development, and second, an extrinsic role whereby Tcf4 dependent signals from the connective tissue fibroblasts promote slow myogenesis and repress developmental embryonic myosin heavy chain expression. Thus, using mouse genetic experiments and in vitro co-cultures we show that Tcf4 and the MCT fibroblasts are crucial for proper muscle maturation, differentiation and function. From these results, we conclude that the connective tissue is not only important for adult muscle structure and function, but is a vital component of the niche within which muscle progenitors reside and is a critical regulator of myogenesis.

P707**Board Number: B1011****The Role of Nonmuscle Myosin II-A and II-B in Coronary Vessel Development in Mice.**X. Ma¹, Y. Uchida², C. Bowen¹, Y. Mukoyama², R.S. Adelstein³;¹Laboratory of Molecular Cardiology, Genetics and Developmental Biology Center, NIH/NHLBI, Bethesda, MD, ²Laboratory of Stem Cell and Neuro-Vascular Biology, Genetics and Developmental Biology Center, NIH/NHLBI, Bethesda, MD, ³Laboratory of Molecular Cardiology, Genetics and Developmental Biology Center, NHLBI/NIH, Bethesda, MD

To understand the functions of the different nonmuscle myosin II isoforms (NM IIs) during mouse heart development we crossed mice expressing cre-recombinase controlled by various promoters with floxed NMHC II-A and II-B mice. Nkx2.5-Cre mediated ablation of NM II-B (B^{Nkx}/B^{Nkx}) in cardiomyocytes caused cytokinesis defects similar to those seen in NM II-B ablated mouse hearts. A^{Nkx}/A^{Nkx} cardiomyocytes on the other hand showed no defects in heart development. Mice ablated for NM II-B by SM22a-Cre in epicardial and myocardial cells (B^{SM22}/B^{SM22}) survived to adulthood. B^{SM22}/B^{SM22} hearts showed marked abnormalities in coronary vessel remodeling. These mice developed arrhythmogenic right ventricular cardiomyopathy (ARVC) and died suddenly at the age of 6 months. B^{SM22}/B^{SM22} mice resemble humans

with ARVC with the exception that they lack fat cell deposition in the myocardium. Defects in coronary vessels were confirmed in mice ablated for NM II-B specifically in epicardial cells by crossing to WT-1-Cre mice however B^{WT}/B^{WT} mice died during embryonic development for unknown reasons. Consistent with abnormal coronary vessel formation, epicardial explants showed that genetic ablation of NM II-B or inhibition of all NM II activity by blebbistatin impaired epicardial EMT. Surprisingly, despite significant expression of NM II-A in epicardial cells, A^{SM22}/A^{SM22} mice showed no obvious heart defects and survived to adulthood. We next ablated NM II in endocardial cells using Tie2-Cre mice. Except for a minor delay in coronary plexus coverage in embryonic hearts, B^{Tie2}/B^{Tie2} mice survived to adulthood with no obvious defects. In contrast A^{Tie2}/A^{Tie2} mice showed defects in coronary vessel formation in embryonic hearts and died during embryonic development. Of note A^{Tie2}/A^{Tie2} embryonic mouse hearts at E14.5 showed marked impairment in coronary coverage over the heart surface indicting a sprouting defect during coronary vessel formation. Furthermore compound Tie2-Cre mice ablated for one allele of NM II-A and both alleles of NM II-B ($A^{+}/A^{Tie2}; B^{Tie2}/B^{Tie2}$) developed a severe defect in coronary coverage suggesting that NM II-B functions when NM II-A expression is limited. These results reveal that NM II-A and II-B function in two different developmental processes during coronary vessel formation in the mouse heart. NM II-A regulates coronary plexus expansion over the heart surface through the endocardial/endothelial cell lineage. NM II-B regulates coronary vessel remodeling through the epicardial derived cell lineage.

P708

Board Number: B1012

VEGF immunoexpression related to the ectomesenchymal cells differentiation into osteoblasts during early development of alveolar process in rat molars.

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The alveolar process is produced by osteoblasts derived from ectomesenchymal (ECM) cells during tooth germ development. As soon as root tooth formation begins, osteoblasts derived from ECM of dental follicle secrete alveolar bone on the inner surface of the alveolar process; this bone layer plays an important role for the maintenance of tooth in the alveolar process. It has been suggested that vascular endothelial growth factor (VEGF) can stimulate osteoblast proliferation and differentiation. Runx2 is a transcriptional factor required for cells differentiate into osteoblasts. Alkaline phosphatase (APh), osterix and osteocalcin (OCN) are considered osteoblast markers. In the present study, we evaluated the immunoexpression pattern of VEGF, Runx2, APh, osterix and OCN by ECM cells in the developing alveolar process. The heads of 16-, 18- and 20-day-old embryos (EM) and 5-, 10- and 15-day-old male rats were fixed and embedded in paraffin. Sections exhibiting ECM surrounding the first upper molar tooth germs were subjected to immunohistochemistry for VEGF, Runx2, APh, osterix and OCN detection; numerical density of immunolabeled-ECM cells was computed. Data were statistically evaluated by Tukey's test ($p \leq 0.05$). Sections of 16- and 20-day-old EM were submitted to von Kossa for detection of calcified bone matrix. In 16-day-old EM, clusters of VEGF-positive ECM cells were observed near to

blood vessels; immunoexpression of Runx2, osterix and APh was also observed in the ECM. von Kossa bone trabeculae surrounding the first molars were observed in the 16- and 18-day-old EM, characterizing the beginning of the development of alveolar process. Osteoblasts adjacent to the bone surface exhibited strong osterix, APh and OCN immunolabeling. However, a significant reduction in the number of osteoblast markers immunopositive ECM cells was verified in the dental follicle from 18-day-old EM to 5-day-old rats in comparison to 16-day-old EM. However, the early formation of tooth root (10-day-old rats) was coupled with a significant increase in the VEGF, Runx-2, osterix and OCN immunolabeled ECM cells in the dental follicle. Thus, the high number of VEGF-immunolabeled ECM cells in the beginning of the formation of alveolar process (16-day-old EM) and alveolar bone (10-day-old rats) suggests a participation of this vascular factor in the process of osteoblast differentiation.

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P709

Board Number: B1013

Rab8a vesicular traffic maintains Wnt activity in intestinal stem cell niche.

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Wnt proteins are a critical group of ligands that drive embryogenesis and maintain adult tissue homeostasis in all animals. Extensive studies using the mammalian intestinal crypt as a model have been conducted to elucidate the role of Wnt signaling in adult stem cell niche maintenance and colorectal tumorigenesis. However, in contrast, little is known about the molecular mechanisms regulating Wnt ligand secretion from the signal-providing cells. We demonstrate that a Ras-like small GTPase, Rab8a directly controls Wnt secretion and signaling in vitro and in mouse intestinal epithelium, contributing to the intestinal stem cell homeostasis. Rab8a knockout intestinal crypts demonstrate significant reduction in canonical Wnt activity that could be partially restored by exogenous ligands or by activating downstream signaling pathway. Despite a significant reduction of the Wnt-producing Paneth cells, an expansion of proliferative transit amplifying cells was detected. Mechanistically, we observed that Rab8a vesicles intersect the traffic of G-protein coupled receptor 177 (Gpr177/Wls), the Wnt-specific transporter. Our study suggested the role of Rab8a in sorting Gpr177-Wnt complexes for exocytosis.

P710**Board Number: B1014****Development and Characterization of an *in vitro* Co-culture Angiogenesis Model Using hTERT Immortalized Cells.**C. Zou¹, C. Zhang¹, I. Oliva¹, M.S. Enuameh¹;¹ACS, ATCC, Gaithersburg, MD

Angiogenesis is the key multi-step physiological process during normal tissue development and is involved in a large number of disease states; an *in vitro* angiogenesis model provides a very useful tool to study this important physiological process. Additionally this model can be used in drug screening applications. Currently, several *in vitro* co-culture models have been developed using primary cells, however, donor variability, low cell quantity per lot and short lifespan of primary cells used for these co-culture models limit their usefulness and consistency.

In this study, we established an *in vitro* co-culture model system using TeloHAEC(an aortic endothelial cell line), and hTERT-MSC cells(adipose derived mesenchymal stem cells); both cell lines were immortalized by hTERT alone and have been well characterized showing the cells retain the most important characteristic of their parental counterparts; when TeloHAEC was co-cultured with BJ primary fibroblast for 14 days in optimized ATCC angiogenesis medium, fine tubular structures were formed as shown by staining with CD31 endothelial cell marker. The tubular length elongated with increasing doses of VEGF stimulation, and the tubular formation can be completely blocked by Suramin or VEGF antibody in a dose dependent manner. Next, we introduced GFP into the TeloHAEC cell line(TeloHAEC-GFP), allowing for real time visualization of angiogenesis when co-cultured with BJ fibroblasts; then another hTERT immortalized cell line, hTERT-MSC, is used to replace the BJ-primary fibroblast in the co-culturing system; results showed that the new model not only can form the tubular structures in less than 7 days instead of 14 days, but also responds effectively to VEGF stimulation and compounds treatments such as suramin, and furthermore, the hTERT-MSC cells which surround the tubular structures have undergone transformation indicated by positive α SMA staining which is a marker of smooth muscle cells, this indicates the system has physiological relevance; most importantly, the tubular structures in this system can be blocked completely with the increasing doses of clinical cancer drugs- such as sunitib, indicating the system is reliable for drug screening purpose. In a word, the co-culture models developed by using hTERT immortalized cell lines described in this report provide a much more consistent and robust *in vitro* co-culture system for studying vascular biology, drug screening and tissue engineering.

P711**Board Number: B1015****Characterization of a Three-Dimensional (3D) Organotypic Skin Model using Keratinocytes and Mesenchymal Stem Cells Immortalized by hTERT.**A. Briley¹, C. Zhang¹;¹ATCC, Manassas, VA

Primary and hTERT immortalized keratinocyte cultures are important cell models for the study of normal and pathological biology of the cutaneous epithelia. Primary keratinocytes can form skin equivalents that mimic the architectural features and behavior of normal skin in a three-dimensional (3D) organotypic culture model in an air-liquid interface (ALI). However, primary keratinocytes have finite lifespan in culture, which greatly restricts their use as in vitro cellular model.

In this study, we compared primary keratinocytes to hTERT immortalized keratinocytes, co-cultured with either fibroblasts or hTERT immortalized mesenchymal stem cells (MSCs). We confirmed that both primary keratinocytes and a hTERT immortalized keratinocyte cell line, Ker-CT are able to fully differentiate into skin equivalents in an ALI 3D culture model, when co-cultured with primary fibroblast or hTERT immortalized mesenchymal stem cells (MSCs). To confirm the functionality of the co-culture models, both primary keratinocytes and Ker-CT ALI co-cultures were subjected to a scratch assay. Re-epithelialization occurred in both cell lines and several markers show increased expression corresponding to crosstalk between keratinocytes and MSC/Fibroblast. The immortality of Ker-CT cell line makes it an invaluable model for the research of keratinocyte biology, as it eliminates the issue of short life span and donor variation seen with primary cells.

P712**Board Number: B1016****Proteomics of mammary epithelial morphogenesis. The role of primary cilia and annexin family proteins.**S. Rossetti¹, W. Bshara¹, J. Reiners¹, F. Corlazzoli¹, A. Miller¹, N. Sacchi¹;¹Roswell Park Cancer Institute, Buffalo, NY

Three dimensional (3D) organotypic models are extensively used to identify the mechanisms of mammary epithelial morphogenesis. The disruption of these mechanisms by different oncogenic insults leads to an atypical mammary epithelial acinar architecture, which is a characterizing phenotype of early breast cancer lesions, such as ductal carcinoma in situ (DCIS). Proteomics analysis of a panel of isogenic clones derived from human mammary epithelial cells let us identify within a common protein signature of aberrant 3D acinar morphology members of two groups of proteins: proteins associated with primary cilia structure and function, and members of the Annexin superfamily. By analyzing different morphogenetic stages of normal 3D mammary development, we found that primary cilia arise in mature 3D acini, where they protrude into the lumen. In contrast, in morphologically aberrant 3D acini, primary

cilia are shorter and show no polarization. Moreover, we mechanistically show that deregulation of two annexin proteins, ANXA2 downregulation or ANXA8 upregulation, is sufficient to disrupt 3D acinar architecture of human mammary epithelial cells. The latter is particularly significant because ANXA8 is far more expressed in DCIS samples relative to normal tissue, and correlates with tumor grade and stage and might qualify as a biomarker of breast cancer tumorigenesis.

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P713

Board Number: B1017

Disruption of periosteal osteochondroprogenitor primary cilia stunts postnatal bone growth.

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Periosteal primary cilia are crucial for proper differentiation and migration of bone and cartilage cells in the embryonic appendicular skeleton, but their influence on these cell populations is surprisingly unexplored in postnatal bone. Primary cilia are non-motile organelles that extend from the apical surface of the cell membrane and are involved in mechano- and chemosensing. Previous studies indicate periosteal primary cilia are responsible for the osteochondroprogenitor nature of periosteal cells and are involved in the migration of osteochondroprogenitors to form the embryonic skeleton. However, these findings are limited to prenatal development due to the embryonic lethality of current mouse models. Because periosteal primary cilia are critical in embryogenesis, we hypothesized that their influence extends into postnatal bone development. Our goal in this study was to evaluate *in vivo* the effects of disrupted primary cilia on the differentiation and migration of periosteal osteochondroprogenitors and resulting endochondral ossification of the postnatal appendicular skeleton. We developed a novel mouse model that allows us to track periosteal progenitor daughter cells and disrupt their primary cilia at developmental time points of our choosing. This model utilizes a periosteal progenitor specific promoter (Prx1) that drives expression of a tamoxifen-inducible conditional Cre recombinase (CreER) and a GFP tag. Recombination at Kif3a, a gene that encodes for a subunit of a ciliary motor protein, is known to disrupt primary cilia. Prx1CreER-GFP; Kif3a^{fl/fl} experimental and Kif3a^{fl/fl} control littermates were injected daily with tamoxifen from postnatal day 5 (P5) until P15. Upon sacrifice, the ulna and radius were dissected and prepared for histochemistry. In mice with disrupted periosteal primary cilia, the proliferation zone contains fewer chondrocytes and the chondrocytes that are present are less densely packed and vertically aligned than those observed in control mice. The hypertrophic zone is a clear boundary between the proliferation and ossification zones in controls, but is less distinct in mice with disrupted cilia. The growth plates are properly formed but the proliferation zones are disorganized and smaller in experimental mice, indicating endochondral ossification is present but stunted when periosteal primary cilia are disrupted. Our data suggest

disrupting postnatal periosteal primary cilia results in a failure to adequately access osteochondroprogenitor stores, resulting in fewer chondrocytes and osteoblasts participating in endochondral ossification. Because primary cilia are sensory organelles involved in Hedgehog and Wnt signaling, it is likely that they facilitate the differentiation of periosteal osteochondroprogenitors. Given the novelty of these findings it is unclear whether periosteal primary cilia are also involved in the migration and proliferation of osteochondroprogenitors, as seen in embryonic studies. Future studies should focus on elucidating the role of periosteal primary cilia in order to manipulate cell stores as a regenerative tool.

P714

Board Number: B1018

Evolutionary, Environmental, and Behavioral, Analyses of Disrupted-in-Schizophrenia-1 (DISC1) in a Conditional Knock-in Mouse.

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Cytogenetics, along with linkage and association studies, implicated Disrupted-in-Schizophrenia-1 (DISC1) as a predisposing risk factor for neuropsychiatric illnesses (Chubb et al, 2008). Additionally, a number of social factors have also been shown to increase the risk of schizophrenia (Thomson et al, 2013). Thus, we hypothesized that aberrant DISC1 function, combined with external environmental stimuli, will affect behavior and protein expression within the hippocampus and prefrontal cortex over a period of time. A three-pronged approach was undertaken. First, the evolutionary relationship of DISC1 across different species was explored. In order to explore DISC1's conservation across species, bioinformatics tools were used to compare nineteen named DISC1 protein sequences chosen from national databases and from nineteen different organisms. The N- and C-terminal regions were shown to be conserved in primates, rodents, and omnivores (with few exceptions). Phylogenetic tree analysis further illustrated the evolutionary relationship of DISC1 in these organisms. Second, we wanted to know if changes in environment could affect protein expression in mutant versus wild type (WT) mice. Following exposure to different environments, animals were sacrificed at ages determined to be important for continued CNS cognitive abilities (Chubb et al, 2008) and analyzed for changes in protein expression. Analysis of protein bands by silver staining disclosed differences in intensity of several gel bands from both the cortex and hippocampus. The proteins in these bands were identified using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). Proteins identified in the cortex, which is involved in cognitive abilities, were alpha-enolase (Eno1) and β -Tubulin (TUBB) while fructose-bisphosphate aldolase C (Aldoc) was identified in bands from hippocampus, an area associated with memory formation. Finally, we wanted to know if different environments adversely affected the behavior of mutant versus WT mice. Data analyses of a forced swim test (FST) showed a statistically significant difference (p

P715**Board Number: B1019****Effects of prolactin, EGF and dexamethasone on mechanism for regulating β -casein expression and secretion.**S. Oyama¹, C. Kuki², H. Kumura², K. Kobayashi²;¹Research Faculty of Agriculture, Hokkaido university, Sapporo, Japan, ²Research Faculty of Agriculture, Hokkaido University, Sapporo, Japan

Mammary alveolar epithelial cells (MECs) synthesize and secrete milk constituents including lactose, milk fat and milk protein during lactation. The milk production ability of MECs is induced by a variety of lactogenic hormones and growth factors. In particular, prolactin, glucocorticoid and EGF have been reported to stimulate milk production in MECs around parturition. However, the regulatory mechanisms of milk production in MECs are still incompletely understood. Therefore, in this study, we focused on regulatory mechanism of β -casein synthesis and secretion by prolactin, EGF and dexamethasone using in vitro milk-secreting model. MECs were isolated from the mammary gland of 8-13-week-old virgin ICR mice and cultured in growth medium without prolactin and dexamethasone. After 6-7 days of culture (approximately 3 days after reaching confluence), MECs were transferred into differentiation medium containing prolactin, EGF and dexamethasone and cultured for 5 days to examine the temporal change of β -casein synthesis and secretory ability. Next, we prepared the incomplete medium, which lacked one of three factors and cultured MECs in each medium for 5 days to evaluate effects of three factors on β -casein expression and secretion in vitro. MEC expressed and secreted β -casein into the medium in the presence of prolactin, EGF and dexamethasone. The immunostaining images showed the subcellular localization of β -casein in endoplasmic reticulum (ER), Golgi apparatus and secretory vesicles in MECs. On the other hand, MECs, which were cultured in the incomplete medium, showed different influences of prolactin, EGF and dexamethasone on β -casein expression and secretion. Beta-casein expression was not detected in the absence of prolactin. Prolactin induced dose- and time-dependent increases in β -casein expression. EGF induced a dose-dependent increase in β -casein expression in the range between 0 and 10 ng/ml although high concentration of EGF (50 ng/ml) inhibited β -casein expression. Dexamethasone also stimulates β -casein expression but didn't show dose-dependent increase of β -casein expression in the range between 1 and 1000 nM. In addition, dexamethasone facilitated β -casein secretion in the presence of prolactin and EGF. These results suggest that prolactin, EGF and dexamethasone stimulate β -casein expression and have differential roles for appropriate β -casein production such as initiation of β -casein expression by prolactin and stimulation of beta-casein secretion by dexamethasone.

P716**Board Number: B1020****Differential roles of prolactin and glucocorticoid in mammary alveolar tight junction formation during lactation in mice.**K. Kobayashi¹, S. Oyama¹, C. Kuki¹, H. Kumura¹;¹Research Faculty of Agriculture, Hokkaido University, Sapporo, Japan

In lactating mammary glands, alveolar tight junctions (TJs) are impermeable and thus allow milk to be secreted from the apical membrane without the leakage of milk components from the lumen into paracellular pathways. The full functional integrity of alveolar TJs is established shortly after parturition and they remain closed throughout lactation. We have previously reported that claudin-3 is one of the major components of mammary alveolar TJs during lactation whereas claudin-4 is down-regulated after parturition until weaning. Thus, it is suggested that claudin-3 and -4 are differentially regulated after parturition to form impermeable alveolar TJs. However, it remains unclear what regulates the expression of claudin-3 and -4 during lactation. In this study, we focused on prolactin/STAT5 and glucocorticoid/glucocorticoid receptor (GR) pathways, which are activated in mammary alveolar epithelial cells (MECs) after parturition and during lactation. Activation of prolactin/STAT5 pathway decreased expressions of claudin-3 and -4 in time- and dose-dependent manners in MECs *in vitro*. In contrast, activation of glucocorticoid/GR pathway increased claudin-3 and -4 in time- and dose-dependent manners. Immunostaining images of claudin-3 and -4 showed that glucocorticoid induced continuous TJs between MECs although prolactin causes discontinuous TJs. Interestingly, co-application of prolactin and dexamethasone specifically decreased the expression of claudin-4. The continuous and clear staining pattern of claudin-3 was observed between MECs with weak staining for claudin-4. We also measured transepithelial resistance (TER) and fluorescein flux across MEC monolayers in the presence of prolactin and/or glucocorticoid. Prolactin induced a decrease in the TER of MECs and an increase in fluorescein flux. In contrast, glucocorticoid induced an increase in the TER of MECs and a decrease in fluorescein flux in the presence or absence of prolactin. These results indicate differential roles of prolactin and glucocorticoid in mammary alveolar TJ formation through regulating claudin-3 and -4 expressions.

P717**Board Number: B1021****Endothelial cell dynamics in Angiogenesis *in vitro*—Anastomosis and Pattern Formation.**M. Shan¹, W.R. Bedell¹, A. Diaz-Santana¹, A.D. Stroock¹;¹Chemical and Biomolecular Engineering, Cornell University, Ithaca, NY

This study dissects *in vitro* the cellular dynamics underlying two stages of angiogenesis: pattern formation during the initiation of sprouting, and vascular anastomosis in the final stages of sprouting.

The heterogeneous differentiation or patterning of neighboring endothelial cells enables the formation of a multicellular sprout; the anastomosis of two vascular lumens enables blood circulation and perfusion to the tissue. These two processes govern the beginning and the end of angiogenesis and are critical targets for the controlling the formation of new vascular networks.

To begin our exploration of endothelial patterning, we analyzed four hypothetical gene regulatory networks (GRNs) suggested by previous experimental studies of endothelial differentiation. We determined the qualitative and quantitative tissue-scale patterning features each GRN would produce should it be the primary mechanism for initial self-assembly in the endothelium. Our analysis suggests that current models endothelial gene regulation can produce multicellular patterns from uniform initial conditions, though the spatial patterns formed are limited to close-packed 'checkerboards' of heterogeneous differentiation that may or may not reflect the earliest patterns of endothelial cell gene expression in response to angiogenic stimuli. We propose additional mathematical and experimental work to clarify the multi-scale gene regulation underlying the initiation of sprouting angiogenesis.

In the study of vascular anastomosis, we exploited a three-dimensional (3D) culture platform *in vitro* to examine the endothelial cell (EC) dynamics during vascular anastomosis and the post events by mimicking angiogenesis and vasculogenesis in parallel. We showed that the cell interactions are present during anastomosis between sprouts formed by angiogenesis from an endothelium and tubes formed by vasculogenesis in the bulk of a 3D matrix. We observed that pericytes were co-localized with the lumen structures, which suggests an important role of pericytes in vessel maturation. We conclude that the occurrence of anastomosis between ECs undergoing sprouting angiogenesis and vasculogenesis can be achieved in this 3D homotypic co-culture platform. We hypothesize that vascular structures act as cellular tracks to promote endothelial cell migration in the process of anastomosis.

Embryogenesis

P718

Board Number: B1022

Cation/proton exchanger 1 protein (Cax1), a maternal-effect regulator of zebrafish cytoplasmic segregation and mRNA localization.

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Early developmental processes are regulated by maternally-provided gene products accumulated in the egg during oogenesis. In the zebrafish, egg activation is followed by cytoplasmic movements toward the animal pole along specific pathways or streamers to form a prominent cytoplasmic domain or blastodisc. This process is called cytoplasmic segregation. The nature and function of maternal factors controlling

cytoplasmic movements, as well as other cellular processes remain largely unexplored in vertebrates. To this end, we are using large-scale forward and reverse genetic screens to identify maternal regulators required to ensure normal zebrafish embryogenesis. We have identified two recessive maternal-effect mutants, *emulsion* (*emn*) and *cax1*, which exhibit defective cytoplasmic segregation. Molecular and cellular approaches reveal that mutant embryos display reduced animal-ward transport of cytoplasm and maternal mRNAs, abnormal streamers and blastodisc formation, altered cleavage pattern and axis determination. Also, the calcium-dependent assembly of parallel arrays of microtubules involved in the transport of maternal determinants appears affected in the mutants. Positional cloning and whole exome sequencing strategies indicate that the *emn* and *cax1* genes encode a Cation/proton exchanger 1 protein (Cax1). Predicted transmembrane segments of Emn/Cax1 suggest that the mutant transcripts would produce a structurally incomplete protein lacking the C-terminal portion. Phylogenetic analysis of protein sequences displays a high level of similarity among unicellular and multicellular organisms, specifically concerning with this part of the protein. Interestingly, homology modeling performed on the C-terminal portion of zebrafish Emn/Cax1 shows a higher sequence similarity to the overall protein structure of bacterial and fungal variants. Thus, our results strongly suggest: 1) a strictly maternal role of Emn/Cax1 controlling early developmental processes, 2) an interdependence between calcium and protons to control cytoplasmic movements, 3) that the gradient of protons across the endomembrane compartments could be utilized to re-uptake calcium after a signaling event and 4) a bifunctional topology of zebrafish Emn/Cax1, with the ion exchanger activity mapped to the C-terminal portion of the protein and the N-terminal portion with a specific function that has not yet been described. Functional studies will allow us: a) to decipher how the *emn/cax1* gene regulates cytoplasmic segregation and mRNA localization, b) Emn/Cax1 kinetics in transport of ions and maintenance of calcium homeostasis, and c) to generate a high-quality structural model to understand the biophysical and molecular basis of Emn/Cax1 function during early vertebrate development.

P719

Board Number: B1023

Dynamics and Shaping of the BMP Signaling Gradient by the BMP Antagonists during DV axial Patterning.

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A gradient of Bone Morphogenetic Protein (BMP) signaling patterns the Dorsal-Ventral (DV) axis of the vertebrate embryo. However, its shape and dynamics during zebrafish DV patterning have not been quantified. Furthermore, how the known extracellular BMP regulators shape the gradient in time and space is not known. To measure the BMP signaling gradient, we quantified the nuclear intensities of fluorescently stained Phosphorylated-Smad5 (P-Smad) in every cell of the embryo at 30-minute intervals from blastula through early gastrula stages. We used automated algorithms to identify the thousands of individual nuclei present at each embryonic time-point, and to measure their corresponding P-Smad

intensities. In WT embryos, we show that the P-Smad gradient intensifies and steepens in the late blastula before stabilizing in the early gastrula. We compared the BMP signaling gradients in WT and BMP antagonist loss-of-function embryos of Chordin (Chd), Noggin (Nog), and Follistatin (Flst) to determine their spatiotemporal functions. Though Chd primarily inhibits BMP signaling, its homologue Sog has been shown to enhance BMP signaling by transporting BMP ligand in *Drosophila* DV patterning and crossvein formation. However, we find that Chd only inhibits BMP signaling laterally in DV patterning in the gastrula. This region-specific inhibition allows Chd to steepen the BMP gradient. In contrast, loss of Nog and Flst have no effect on the gradient. However, the loss of all three antagonists causes a massive embryo-wide increase in BMP signal, far exceeding the lateral-only increase seen when Chd alone is absent. To elucidate mechanisms explaining these results, we generated a mathematical model of the system and screened thousands networks consisting of different rates of production, diffusion, degradation, and binding between the BMP ligand, Chd, Nog, Flst, and Tolloid/Bmp1a. Our results suggest that: 1) in a WT system, Chd functions as the primary sink for BMP ligand. 2) When Chd is absent, Nog and Flst take over as the primary sink for the BMP ligand. 3) When all three antagonists are absent, no extracellular sink exists, and BMP signaling increases embryo-wide.

P720

Board Number: B1024

Split top: A Maternal Regulator of Dorsal-Ventral Patterning and Cell Migration in Zebrafish.

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Little is known about the maternal factors that function in body axis formation during vertebrate embryonic development. To identify these factors, our lab has performed a recessive maternal-effect mutagenesis screen in the zebrafish and found a number of mutants with defects in early developmental processes, including early morphogenesis and body axis formation. One such mutant, *split top* exhibits a dorsalization of the embryonic axis. Clutches of embryos from *split top* mutant mothers are characterized by the five classic dorsalized phenotypic classes, as well as some additional defects. The mutant embryos show an expansion of dorsal markers and a corresponding reduction in ventral markers during gastrulation indicative of dorsalization. The dorsalization defects can be rescued by misexpression of either BMP2 or BMP7 ligands, or by derepression of BMP signaling by knockdown of BMP antagonists. The additional defects appear to be the result of altered morphogenesis, including defects in epiboly progression, the process by which the blastoderm cells migrate over and surround the yolk. Mutant embryos display altered microtubule and actin cytoskeletal networks in the yolk cell, which can account for the epiboly defects observed. *split top* mutant embryos also appear to be defective in the cell movement process of convergence and extension. We mapped the *split top* mutation to

chromosome 17, and have identified a candidate gene through RNA-Seq and traditional positional cloning methods.

This work was supported by NIH grant R01-GM56326, NIH training grant T32HD007516, and the PENN-PORT training program.

P721

Board Number: B1025

Identification and characterization of paternal-effect lethal mutants in *C. elegans*.

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Infertility affects ~15% US couples and ~25% of couples in developing countries. In many cases the underlying cause of infertility is unknown. While the field of reproductive medicine is rapidly growing, in the cases where the cause of infertility is unknown the needs of these couples are not being addressed. Therefore, understanding the genetic and molecular basis of fertilization and early embryogenesis is important. At fertilization the oocyte and the sperm fuse, restoring the somatic chromosome number and initiating zygotic development. Despite both deriving from meiosis, the morphologies of oocytes and sperm are distinct. Oocytes are large, sedentary cells that provide a haploid genome and large stockpiles of maternal RNA and proteins necessary for early embryogenesis. Sperm are small, motile cells streamlined for fertilization but also play essential roles in early development. The sperm provides a haploid genome, centrosomes, and initiates the embryonic program. There is evidence that sperm contain other factors required for embryogenesis. Absence of these paternally provided components results in paternal-effect embryonic lethality (PEL). In *C. elegans*, *spe-11* is the only strictly PEL gene. SPE-11 is a novel protein supplied by the sperm. The absence of functional SPE-11 results in embryonic failure at early stages. I am interested in the further characterization of SPE-11 through structure function analysis. I have recently demonstrated that the extreme C-terminus of SPE-11 is important for the production of viable embryos.

In addition to *spe-11*, I have identified another novel PEL mutant. This mutant was previously identified in a screen for maternal-effect embryonic lethal mutants. However, embryonic lethality of this mutant can be rescued by mating to wild-type males indicating a potential sperm defect. Analysis of this mutant showed that it has >95% rescue of the embryonic lethality phenotype upon mating to wild-type males. Initial analysis of this mutant, *mel-15*, confirms that sperm from *mel-15* mutant males produce dead embryos, even when fertilizing wild-type oocytes. In addition, characterization of early embryonic events indicates fertilization is successful and that sperm components (e.g. centrosomes) are transmitted to the embryo, but that *mel-15* sperm lack DNA. I am currently in the process of determining the molecular identity of *mel-15* and where in the meiotic cell cycle sperm DNA is being mis-segregated.

P722**Board Number: B1026****Studying The Regulation of Temporal Patterning of String Transcription in Early *Drosophila* Cell Cycles.**A. Momen Roknabadi¹, S. Di Talia², E. Wieschaus¹;¹Department of Molecular Biology, Princeton University/HHMI, Princeton, NJ, ²Department of Cell Biology, Duke University Medical Center, Durham, NC

An embryo develops in a highly intricate spatiotemporal pattern. Although there is much understood about the mechanisms underlying spatial patterning throughout the life of an organism, we still do not know whether there are additional regulators that modulate the temporal patterning in various key biological processes, including but not limited to cell cycle or cell differentiation. In this study, we aim to understand, at a molecular level, how a temporal pattern can be achieved. Here, we have chosen as a model the spatiotemporally patterned 14th mitosis in the early *Drosophila* embryo. This pattern is regulated by the transcription of the *cdc25* homolog, *string* (*stg*), which drives the mitosis in 25 distinct and highly reproducible mitotic domains (MD). We hypothesize that the temporal patterning is controlled by how fast the rate-limiting regulators accumulate to the level required for triggering *stg* transcription globally or in each specific MD. Therefore we predict that by changing the dosage of *stg* regulators, we can change the observed temporal pattern. Using a whole-genome screen we have identified several rate-limiting effectors that encode the underlying molecular clock for specific domains.

In this study, we use heterozygous deficiency lines to identify dosage dependent effectors that regulate the temporal expression of *stg* in MD 1 and 2. Heterozygote embryos develop with normal spatial pattern, thereby allowing us to study the effect of the candidate regulators. In this screen, we count the number of cells undergoing mitosis in MD 1 and 2 in the wild type (WT) and the heterozygote deficiency lines. Using this measurement, we compute the relative timing of the onset of mitosis in MD 1 and 2. In theory, a rate-limiting factor may either act as a general factor that sets up the timing globally throughout the embryo, or acts more locally in a domain-specific manner. Here, we have observed hardly any evidence for the global regulators, yet we have identified several factors that regulate timing of specific domains. For example the relative time difference of mitosis between the MD1 and 2 from 3 minutes in WT is changed to a simultaneous division by halving the dosage of *buttonhead*. The results of this screen suggest that every domain has its own clock. The majority of the identified domain-specific regulators are known patterning genes that also regulate the spatial patterning in the corresponding mitotic domain. Therefore, we conclude that the transcriptional factors that confer the spatial pattern are also the rate-limiting factors that set the temporal pattern in development. The rate at which these spatial regulators accumulate through time comprises the molecular clock that we observe at different stages of develop

P723**Board Number: B1027****Investigating the importance of size scaling relationships in *Xenopus* development.**R. Gibeaux¹, R. Heald²;¹University of California, Berkeley, Berkeley, CA, ²Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

Determining how size is controlled is a fundamental question in biology that is poorly understood at the organism, cellular and subcellular levels. The two frog species *Xenopus tropicalis* and *Xenopus laevis* differ in size at all three of these levels. Diploid wild-type (WT) *X. tropicalis* has 20 chromosomes and is smaller (~4 cm adults), whereas diploid WT *X. laevis* has 36 chromosomes and is bigger (~10 cm adults). Scaling at the organismal and genome levels is accompanied by differences in the size of the egg as well as nuclei and mitotic spindles formed in egg extracts. Despite these size differences, the close phylogenetic relationship between these two species allows the production of hybrid embryos by cross-fertilization. Taking advantage of the viable hybrid produced when *X. laevis* eggs are fertilized by *X. tropicalis* sperm (*lexts*), we are investigating how an embryo that is initially identical in size and developmental rate to that of *X. laevis* generates a hybrid frog closer in size to *X. tropicalis*. Using live embryo imaging to monitor early cleavage phases, and immunofluorescence to determine nucleocytoplasmic and genome-spindle size ratios, and by comparing *lexts* hybrids to diploid and haploid *X. laevis*, we are assessing the importance of scaling relationships in embryo development. In parallel, we are investigating the reverse hybrid generated by the fertilization of *X. tropicalis* eggs with *X. laevis* sperm (*texls*), which is not viable and dies at the late blastula stage. We observed gross mitotic defects prior to embryo death and are testing the hypothesis that *texls* hybrid death is caused by scaling defects that lead to chromosome segregation errors, resulting in large-scale apoptosis.

P724**Board Number: B1028****Centrin-2 mediated regulation of FGF/FGFR gene expression in *Xenopus*.**M. Winey*¹, J. Shi¹, Y. Zhao¹, T. Vonderfecht¹, M. Klymkowsky*¹;¹MCDB, University of Colorado-Boulder, Boulder, CO

Centrins (CETNs) are highly conserved, widely expressed and multifunctional Ca²⁺-binding eukaryotic signature proteins best known for their roles in ciliogenesis and to a lesser extent as critical components of the UV DNA damage repair system. The clawed frog *Xenopus laevis* contains three *Cetn* genes, *Cetn2*, *Cetn3*, and *Cetn4* whose RNAs are provided maternally and throughout embryogenesis. Using ectodermal and ectodermal/mesodermal explants and a range of analytical methods, we have characterized the phenotypes associated with morpholino-based loss of *Cetn* function. In addition to defects in the formation of epidermal multiciliated cells, we have identified a previously unreported *Cetn2*-specific function, namely in the regulation of FGF signaling, specifically through the regulation of

FGF and FGF receptor RNA levels. Cctn2 was found associated with RNA polymerase II binding sites of the CETN2-regulated FGF8 and FGFR1a genes, but not at the promoters of genes whose expression was not altered by down-regulation of CETN2. We are in the process of defining the universe of genomic targets associated with Cctn2 and its associated proteins. These observations point to a previously unexpected role of CETN2 in the regulation of gene expression and embryonic development that could have implications in a range of cellular systems.

P725

Board Number: B1029

Regulation of BMP signaling by Pitx1 during the formation of Xenopus cement gland.

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The cement gland is one of the first fully functional tissues to differentiate during the development of the frog *Xenopus laevis*. It secretes mucus to help the developing tadpole attach to solid supports and live in relative safety. The gland develops from the outer layer of anterior ectoderm between the neural plate and non-neural ventral ectoderm. As a marker of anterior ectodermal fate, the cement gland is a useful model to study how different signaling pathways are regionally integrated in the embryo. It has previously been proposed that an intermediate level of Bone Morphogenetic Protein (BMP) signaling promotes cement gland formation. Additionally, several transcription factors have been related to cement gland differentiation. One of them is Pitx1, a homeobox gene which is both sufficient and necessary to induce ectopic cement gland formation. However, the mechanisms by which Pitx1 function in this context remains obscure. We hypothesized that the appropriate level of BMP signaling within cement gland primordium is regulated by Pitx1. Our lab found that injection of synthetic Pitx1 messenger RNA into the animal pole of early *Xenopus* embryos not only leads to the induction of cement gland markers, consistent with previous studies, but also suppresses BMP-responsive target genes in a dose dependent manner. A chimeric protein, consisting of the full length Pitx1 fused to a VP16 activation domain, also inhibits BMP targets, while introduction of a Pitx1-Engrailed repressor domain fusion protein does not significantly affect expression of these genes. These results suggest that Pitx1 activates downstream targets to partially suppress BMP signaling, leading to cement gland formation. Taken together, this study links Pitx1 to regulation of BMP signaling during the formation of this anterior-most ectodermal organ.

P726**Board Number: B1030****microRNA-31 Regulates Skeletogenesis of the Sea Urchin Embryo.**N. Stepicheva¹, J.L. Song¹;¹Biological Sciences, University of Delaware, Newark, DE

The microRNAs (miRNAs) are small non-coding RNAs that regulate the translation and stability of target mRNAs. Recently miR-31 has been found to modulate bone formation. It regulates osteoblast differentiation by targeting Osterix and Satb2 and osteoclast activity by inhibiting RhoA. The sea urchin miR-31 has the same sequence as in human, and our work indicates that miR-31 plays a conserved regulatory role in skeletogenesis of the sea urchin. Upon miR-31 knockdown (KD), sea urchin embryos have a significant decrease in primary skeletogenic mesenchyme cells (PMCs), leading us to hypothesize that miR-31 regulates the movement and specification of PMCs. miR-31 KD embryos have abnormal localization of PMCs, as well as highly irregular and extensive connections among the PMCs. Using luciferase reporter constructs and site-directed mutagenesis, we identified Pmar1, Alx1 and Cyp1 within the skeletogenic gene regulatory network to be directly post-transcriptionally regulated by miR-31. Pmar1 indirectly activates Alx1, a key skeletogenic transcription factor that activates PMC-specific genes such as Cyp1. To test the effect of miR-31 regulation on Alx1, we used miRNA target protector morpholinos to mask the two miR-31 regulatory sites within the Alx1 3'UTR and observed PMC mislocalization. Based on our work in the sea urchin, we predict that the vertebrate Alx1 may be regulated by miR-31. Alx1 mediates the fusion of frontonasal, nasomedial, nasolateral, and maxillary processes during early embryogenesis. Alx1 autosomal recessive mutations in vertebrates have frontonasal dysplasia. Bioinformatic analysis identified two miR-31 binding sites within the mouse Alx1 3'UTR, suggesting that miR-31 has an evolutionarily conserved role in regulating the Alx gene family. Our study contributes to the understanding of miR-31 function during early embryogenesis and suggests that the sea urchin animal model can be used to discover conserved miR-31 functions that may be shared by vertebrates.

P727**Board Number: B1031****Role of Cdc42 in the morphogenetic events of the sea urchin embryo.**S.P. Sepulveda¹, J. Hernandez¹, A. Ellis¹, A. Meyer-Miner¹, R. De La Rosa¹, C.B. Shuster¹;¹Biology Department, New Mexico State University, Las Cruces, NM

During gastrulation, changes in cell shape, adhesion and motility drive the formation of the primary germ layers. In the sea urchin, a complex gene regulatory network has been described that drives the specification of the mesoderm and endoderm. However, far less is known about how these specification events contribute to the actual morphogenetic rearrangements during gastrulation. The Rho-family GTPases Rac, Rho and Cdc42 have been shown to be fundamental for these processes in a number of model systems, and indeed, work in other labs has implicated RhoA in the formation of the primary

invagination during sea urchin gastrulation. Given the many advantages of the sea urchin as a model organism for developmental biology, we aim to better characterize the role of Cdc42 in the sea urchin embryo. Using a combination of live cell probes and immunofluorescence, we have identified Cdc42 as a potential regulator of Primary Mesenchymal Cell (PMC) motility and skeletogenesis. PMCs form the spicules of the larvae that give rise to the larval skeleton, and Cdc42 was enriched in PMCs following ingress. Further, inhibition of Cdc42 using small molecule inhibitors had no effect on the PMC epithelial to mesenchymal transition, PMC ingress and fusion. However, Cdc42 inhibition blocked filopodial extension, PMC alignment and spicule deposition. Similarly, inhibition of Cdc42 expression using anti-sense morpholinos showed a delay in PMC ingress. Additionally, PMCs did not exhibit their stereotypical alignment in morphant embryos and failed to form spicules. Lastly, the archenteron failed to extend in Cdc42 morphants, suggesting a possible role for Cdc42 in convergent extension and gut formation. Together, these results suggest that while Cdc42 may be dispensable for establishment of epithelial polarity and PMC ingress in the early embryo, Cdc42 plays a specific role in skeletogenesis, and may also be instrumental in formation of the primordial gut.

P728

Board Number: B1032

Visualization of an Adenosine 5'-triphosphate levels in mouse embryo.

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Adenosine 5'-triphosphate (ATP) is produced in energy metabolism, is the major energy currency in animal and plays an important role in many biological processes. Recent research provides real-time monitoring system of ATP levels inside individual living cells. To monitor the ATP levels in mouse, I generated the transgenic mouse using this system. I examine the ATP levels in developmental stage of mouse embryo. In cleavage stage, ATP levels around nuclear are lower than that in marginal zone until 2 cell stage. In gastrulation stage, ATP levels are high in specific region, organizer and primary heart field. To understand the role of high ATP, I examined the inhibition of glycolysis and/or oxidative phosphorylation by chemical inhibitor. In the result, oxidative phosphorylation would regulate the axis formation and heart formation. I would like to discuss about recent data.

P729**Board Number: B1033****Effects of dynamic myosin phosphorylation on tissue mechanics and integrity during apical constriction.**C.G. Vasquez¹, A.C. Martin¹;¹Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA

Embryonic development requires coordinated cell shape changes to induce global tissue shape change. In the *Drosophila* embryo, apical constriction of a strip of ~1,000 cells along the ventral midline results in higher tension oriented along the long axis of the embryo, relative to the short axis, and higher constriction directed along the short axis, compared to the long axis. This force asymmetry is associated with the formation of a long narrow furrow (ventral furrow) along the length of the epithelium. Apical constriction in ventral furrow cells is driven by cycles of assembly and disassembly of the contractile motor non-muscle myosin II (Myo-II); these cycles (pulses) result in step-wise constriction of the cells, which is staggered between neighboring cells. A still unanswered question is why cells undergo step-wise rather than continuous contraction during tissue morphogenesis. We have found that Myo-II pulses are spatially and temporally associated with pulses of apical Rok suggesting that Myo-II phosphorylation organizes contractile pulses. Mutants that mimic Myo-II light chain phosphorylation or depletion of myosin phosphatase inhibit Myo-II contractile pulses, disrupting both actomyosin coalescence into apical foci and cycles of Myo-II assembly/disassembly. Coupling dynamic Myo-II phosphorylation to upstream signals organizes contractile Myo-II pulses in both space and time. Mutants that lock Myo-II in a specific phosphorylation state undergo a more continuous, rather than incremental, apical constriction. A tissue-scale consequence of continuous constriction is that these mutants fail to maintain intercellular actomyosin network connections during tissue invagination, suggesting that Myo-II pulses are required for tissue integrity during morphogenesis. In addition, we find that cells expressing phosphomimetic Myo-II constrict isotropically, without directionality. Thus, it is possible that Myo-II dynamics are required to generate or respond to patterns of epithelial tension during tissue invagination, ensuring effective cell invagination without compromising tissue integrity. These results provide a mechanism and a function for contractile pulses, suggesting that proper Myo-II regulation is required to ensure appropriate tissue movements during morphogenesis. To further investigate how Myo-II regulation affects the mechanical properties of the ventral furrow tissue, we are performing laser ablation experiments to compare the response of wild-type and Myo-II phospho-mutant embryos.

P730**Board Number: B1034****Functional Epigenomics and Evolutionary Diversification of Mammalian Pre-implantation Blastocysts and Male Fertility.**S. Kwok¹, R. Oliveira², A. Moura², A. Kaya³, A. Perkins⁴, R. Wills⁵, E. Memili⁶;

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Oocyte fertilization by fusion with a spermatozoon initiates embryogenesis and further development. Although both maternal and paternal epigenetic factors have been found to play a role in gamete fertility, embryo viability, and embryo survival, the exact mechanisms regulating maternal:embryonic interaction and embryo development are unclear. This gap in the knowledge base is impeding advances in the science of early mammalian developmental, including fertility prediction and improvement. The objectives of this study were to ascertain cellular characteristics of individual bull sperm samples of distinctly different fertility levels, and to investigate functional epigenomic networks of pre-implantation blastocysts in mice, humans, and cattle. To achieve these objectives we used a combination of scanning electron microscopy (SEM) and ImageJ, and computer-assisted sperm analysis (CASA) for the bull sperm investigation. In order to investigate the blastocyst epigenomic regulation, the networking tools MetaCore and AmiGO were used to examine the expressed epigenome, focusing on DNMT3L, BMI1, and L-selectin to further explore blastocyst epigenetic regulation by DNA methylation and chromatin remodeling, and embryonic adhesion to the mother, respectively. Current findings indicate that there are differences between the individual bull sperm samples in both morphology and function, and in sperm velocity. Furthermore, DNMT3L, BMI1, and L-selectin are interconnected and highly conserved between mice, humans, and cattle. Individual sperm samples from two bulls with high and low fertility scores (5.6 and -14.7) show significant differences in sperm head length (0.8845 μ M) and width (-0.1366 μ M), and tail length (1.6427 μ M). CASA results of sperm from 10 bulls indicate a positive correlation between fertility score and straight line velocity ($p=0.0442$, $r=0.64$). Sequences for DNMT3L, BMI1, and L-selectin show sequence overlaps 51.145%, 95.706%, and 65.065% between all three species, respectively. The findings are significant because they illuminate cellular mechanisms regulating sperm fertility and epigenetic mechanisms regulating implantation of conceptus to endometrium. The knowledge can be used to improve assisted reproductive technologies.

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P731**Board Number: B1035****Dunk stabilizes the tensile actomyosin network at the leading edge of the cleavage furrows during *Drosophila* cellularization.**B. He¹, A.C. Martin², E. Wieschaus³;¹Department of Molecular Biology, Princeton University, Princeton, NJ, ²Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA, ³Department of Molecular Biology, Princeton University/HHMI, Princeton, NJ

In *Drosophila*, the formation of the cellular blastoderm is achieved by cellularization, during which plasma membrane invaginates from the surface of the embryo and partitions the syncytial nuclei into individual cells. During cellularization, a dynamic network of actin-myosin localizes at the basal leading edge of the cleavage furrows (“furrow canals”) and subsequently reorganizes into individual contractile rings that pinch off the cell basally at late cellularization. Here we describe the characterization of novel gene *dunk*, mutation in which disrupts the proper organization of the actin-myosin network at the furrow canals. Using laser ablation, we find that basal myosin is already under tension while it organizes into an interconnected network. Mutation in *dunk* leads to disruption of the network and causes myosin to deplete from the edges and accumulate at the vertices. This in turn leads to a delay in the subsequent formation of myosin rings and causes defect in their morphology. The expression of *dunk* is restricted at the blastoderm stage, and its protein product co-localizes with myosin at the furrow canals. The localization pattern and the mutant phenotype suggest that *dunk* directly involves in stabilization of the actomyosin network at the basal cortex during early cellularization. Computer simulation demonstrates that vertex-accumulation of myosin in *dunk* mutant embryos can be recapitulated by a uniform myosin loss coupled with a positive feedback mechanism that prevents myosin loss upon myosin contraction. We propose that Dunk promotes homogeneous myosin distribution by preventing myosin loss, therefore help to maintain basal contractility symmetry at the invagination front.

P732**Board Number: B1036****A Rho-GAP regulates two key processes during *C. elegans* embryonic morphogenesis.**A.G. Wallace¹, M.C. Soto¹;¹Rutgers - Robert Wood Johnson Medical School, Piscataway, NJ

Rearrangement of the actin cytoskeleton is important during embryonic morphogenesis. In the *C. elegans* embryo, cell migrations that occur during morphogenesis are coordinated by tight regulation of actin dynamics controlled by axonal guidance signals. Previous work in our lab have shown that axonal guidance signals UNC-40/DCC, SAX-3/Robo, and VAB-1/Eph regulate the actin cytoskeleton through a pathway controlled by the WAVE/SCAR complex which is modulated by the CED-10/Rac1 GTPase.

Subsequently, we performed an RNAi screen for regulators of WAVE by screening for enhancers of *unc-40* embryonic lethality. Our screen identified HUM-7, which is the *C. elegans* homolog of the vertebrate Myosin IX gene. Loss of HUM-7 alone results in a small percentage of dead embryos, most of which have morphogenesis defects similar to what is seen in WAVE mutants. Like its vertebrate homolog, Myo9, HUM-7 has a myosin domain in its head and a RhoGAP domain in its C-terminus. The presence of the RhoGAP domain combined with *wve*-like morphogenesis phenotypes, led us to predict that HUM-7 is functioning as a GAP for CED-10 during embryogenesis. To test this hypothesis, we analyzed genetic and RNAi doubles of *hum-7* and hypomorphic alleles of three *C. elegans* GTPases, RHO-1, CDC-42 and CED-10 or their regulators. The results did not support our predictions since HUM-7 genetically behaved like a GAP for RHO-1 and CDC-42 but not for Rac1. These results were further supported by protein-protein interaction assays that showed binding between the GAP domain of HUM-7 and the GTP-loaded forms of RHO-1 and CDC-42 but no binding to Rac-1. Similar to other known RHO-1 pathway genes, loss of HUM-7 causes defects in seam cell constriction, which is a crucial process in the formation of the worm from the embryo. Live imaging of actin dynamics in the *C. elegans* embryo shows that loss of HUM-7 results in increased levels of actin and increased actin dynamics at the leading edge of migrating cells. This suggests that HUM-7 is needed to properly organize actin events that occur during embryonic morphogenesis. In addition, these results suggest a role for RHO-1 in the regulation of actin-based cell migrations that occur during embryonic morphogenesis. Overall, these results suggest that HUM-7 has a role in two key events that occur during morphogenesis, the induction of cell migrations and elongation.

P733

Board Number: B1037

Physical Properties of P-granule Components.

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We study the proteins that comprise the major constituents of liquid droplets called “p-granules”. During the one-cell stage of *Caenorhabditis elegans* development, these p-granules preferentially form on the posterior side of the cell and remain there preferentially as the cell divides leading to the majority of droplets remaining in one of the daughter cells. This process repeats during subsequent cell divisions with, again, the majority of droplets residing in one cell. When this process concludes and the worm continues to develop, the cell containing the p-granule droplets will eventually become the gonad of the adult worm. Previous work has suggested that the spatial segregation of p-granules that occurs at each stage is the result of a phase separation analogous to classic liquid-liquid demixing. We study this process under various buffer conditions *in vitro* by using a model system consisting of purified p-granule components. We find that these components can form liquid droplets under physiological conditions which resemble p-granules. We further report on the physical properties of these liquids and how they

compare to their in vivo counterparts. In particular, we measure their surface tension and viscosity. We find surface tensions around 10^{-7} Nm and viscosities much higher than that of water.

P734

Board Number: B1038

A model-systems approach to coral cell biology.

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Coral reefs are the most biodiverse marine ecosystems and of high ecological as well economic importance. Corals (phylum Cnidaria) thrive in very nutrient-poor waters and their immense productivity strictly depends on a functional symbiosis between corals and dinoflagellates (genus *Symbiodinium*). The photosynthetic symbionts reside within the gastrodermal host cells and transfer energy to the host. Despite the importance of coral reefs we only have limited knowledge about the cellular basis of symbiosis establishment, maintenance and breakdown (also known as “coral bleaching”). The lack of cellular information about the ecologically critically symbiosis is mainly due to the fact that corals are not very suited as laboratory model organisms allowing cellular and molecular analysis. Here we introduce *Aiptasia*, a marine sea anemone, as an emerging laboratory model organism to analyze cnidarian-endosymbiosis. *Aiptasia*, just as many reef-building corals acquires symbionts during planula larval stages from the environment anew each generation. To develop *Aiptasia* into a model system allowing analysis at the cellular and molecular level, a detailed description of the cellular processes involved in symbiosis establishment and maintenance is required as well the development of functional tools. To date, we have generated a detailed description of *Aiptasia* embryonic development and different planula larval stages. Moreover, we have analyzed when and where symbionts are intracellularized and find that *Aiptasia* larvae take up symbionts at a constant rate as early as two days 2 post fertilization (pf) until ten days pf, but uptake efficiency increases thereafter. Accordingly, the region of efficient symbiont uptake changes over time. Initially (day 2 - day 8 pf), symbionts are predominantly taken up in the aboral region of the gastric cavity, while later the uptake region expands to the more oral areas of the cavity. Moreover, we have developed in situ hybridization protocols to analyze gene expression in *Aiptasia* planula larvae and are currently developing further techniques to extend the tool-kit for cellular and molecular analysis.

Pathogen Entry and Intracellular Trafficking

P735

Board Number: B1040

Junin virus DC-SIGN- mediated entry.

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Virus entry and dissemination can be mediated by calcium dependant c-type lectines as DC-Specific ICAM-3 grabbing non-integrin (DC-SIGN), which is a member of a family of receptor that recognizes carbohydrates structures. In previous studies we provided evidence that hDC-SIGN recognizes and internalizes the New World arenavirus Junin (JUNV), etiologic agent of hemorrhagic argentinian fever, which presents highly glycosylated proteins in its envelope. In the present study we provide evidence of the interaction between hDC-SIGN and JUNV, and characterize the early stages of hDC-SIGN-mediated entry. NHI3T3 and NIH3T3-hDC-SIGN cell were infected in presence or absence of EGTA or the carbohydrate bynding agent Griffithsin (GRFT). Both compounds inhibited infection in cultures that express the human lectin. To better understand how the lectins are involved in virus internalization, NIH3T3 cells were transfected with expression plasmids encoding hDS-SIGN wild type or mutants of the conserved cytoplasmatic motifs: dileucine (LL), and triacid cluster (EEE) which are believed to be involved in the regulation of ligands uptake, and trafficking respectively. Our results show that mutations in the cytosolic domain of DC-SIGN impair its ability to enhance JUNV infection. We used compounds that specifically blocked different entry pathways to determine if the virus endocytic route is affected when lectins are expressed in the cell surface. Our results show that virus infection is inhibited with dynasore (DYN), which inhibits the dynamin II-dependent endocytosis, Chlorpromazine (CZ), that inhibit the clathrin- dependent endocytosis and methyl-B-cyclodextrin (MBCD) which inhibit the cholesterol-dependent endocytosis. Using plasmids codifying proteins involved in the endocytic pathway, or its dominant negative forms, we determine cellular proteins and compartments involved in virus entry in the presence of hDC-SIGN. Our studies show that JUNV infection depends on EPS15 and Dynamin-2, both involved in clatrhin-mediated endocytosis. As many entry pathways are dependant of cell cytoskeleton, we studied the rol of microfilaments and microtubules during early virus infection. Inhibition of infection was observed when cells were treated with LatrunculinA which disrupts cortical and cytoplasmic microfilament whereas cytochalasin D, that affects cytoplasmic microfilaments did not inhibit JUNV multiplication. In contrast, drugs that depolymerize microtubules did not show to affect JUNV multiplication. Taken togheter our results demonstrate that JUNV interacts with hDC-SIGN and that JUNV internalization is dependent on clathrin-mediated endocytosis. In addition JUNV infection in the presence of the human lectin depends on cholesterol and intact cortical actin filaments.

P736**Board Number: B1041****HIV-1 antagonism of Tetherin-mediated restriction of macrophage-T cell virus transmission.**S. Giese¹, M. Nijmeijer¹, M. Mazzon¹, M. Marsh¹;¹MRC/UCL Laboratory for Molecular Cell Biology, London, United Kingdom

Bst-2/Tetherin is a cellular protein that inhibits the release of enveloped viruses by physically tethering them to their host cells. A growing body of evidence suggests that the efficient counteraction of Tetherin was a crucial factor for the pandemic spread of the main (M) group human immunodeficiency viruses type 1 (HIV-1). Here we show that in primary macrophages, Tetherin localises to the plasma membrane, the trans-Golgi network, as well as intracellular plasma membrane-connected compartments (IPMCs), which serve as the preferred sites for HIV assembly and budding. Upon infection, Tetherin retains nascent HIV particles in IPMCs, which may cause their passive expansion, but the restriction factor is not required for the formation of the membrane compartments *per se*. In addition to diminishing virus release, we demonstrate that Tetherin inhibits the direct cell-cell transmission of HIV from macrophages to T cells, apparently by promoting the transfer of infectious virus clusters to a limited number of target cells. The viral accessory protein Vpu efficiently counteracts Tetherin in macrophages and thereby overcomes the restriction on cell-free and cell-cell HIV spread.

During SIV/HIV evolution, different viral proteins have adapted to antagonise the Tetherin proteins of their respective hosts, but all M group HIV-1 strains are thought to exclusively rely on Vpu to overcome human Tetherin and ensure their efficient replication. We show that at least one M group HIV-1 uses a viral protein other than Vpu to reduce the levels of Tetherin at the cell surface.

Overall, we show that Tetherin, one of the most potent restriction factors identified to date, inhibits HIV spread from macrophages regardless of the mode of transmission, and that HIV-1 proteins other than Vpu may have anti-Tetherin activity.

P737**Board Number: B1042****Calcium signaling mediated influenza virus entry into host cells.**Y. Fujioka¹, M. Tsuda², A. Nanbo¹, T. Hattori³, J. Sasaki⁴, T. Sasaki⁵, T. Miyazaki^{3,6}, Y. Ohba¹;¹Dep Cell Physiol, Hokkaido Univ Grad Sch Med, Sapporo-shi, Japan, ²Dep Cancer Pathol, Hokkaido Univ Grad Sch Med, Sapporo-shi, Japan, ³Res Ctr Zoonosis Cntl, Hokkaido University, Sapporo-shi, Japan, ⁴Akita Univ Grad Sch Med, Akita, Japan, ⁵Akita Univ Grad Sch Med, Akita-shi, Japan, ⁶Hokkaido Univ Inst Genet Med, Sapporo-shi, Japan

We have reported that Ras-PI3K signaling mediates endocytosis, and influenza viruses exploit this pathway to expedite their efficient incorporation into cells. In this study, we explored in detail the

mechanism by which Ras is activated upon infection with influenza. Because it is well established that Ca^{2+} signaling converge to activate Ras, Ca^{2+} concentration before and after viral infection was analyzed; Cameleon, a FRET based Ca^{2+} sensor showed that robust oscillations in the intracellular calcium concentration were induced by viral infection. Given that buffering Ca^{2+} abolished the virus-dependent Ras activation, Ca^{2+} transmits signals to activate Ras upon infection. We also revealed that RhoA and Ca^{2+} constitute a positive feedback loop, with RhoA not only being activated by Ca^{2+} , but also inducing subsequent $[\text{Ca}^{2+}]_i$ elevation during virus infection, which thereby activates endocytosis. Taken together, signaling mediated by RhoA and Ca^{2+} is demonstrated as a key host-oriented mechanism for viral entry. We further performed high-speed imaging experiments with fluorescently labeled virus particles in order to gain further insight into the spatiotemporal dynamics of Ca^{2+} responses upon virus entry. Immediately after infection, localized and modest Ca^{2+} elevations, prior to the aforementioned robust Ca^{2+} release from ER, were detected in the regions where labeled particles were adsorbed. Moreover, sialidase treatment suppressed virus-dependent such Ca^{2+} increases. Collectively, our data indicate that the localized Ca^{2+} elevation is accounted for by sialylated cell surface protein(s), which may in turn evoke subsequent robust Ca^{2+} signaling during virus infection.

P738

Board Number: B1043

Adaptor protein Sorting Nexin 17 is involved at different stages of the human papillomavirus trafficking.

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The Human Papillomavirus (HPV) L2 capsid protein plays an essential role during the early stages of viral infection, but the molecular mechanisms underlying its mode of action remain obscure. We have identified the adaptor protein, Sorting Nexin 17 (SNX17) as a strong interacting partner of HPV-16 L2. This interaction occurs through NPxF/Y phosphotyrosine-binding domain, which is present in all papillomavirus (PV) types analysed. Using mutants of HPV-16 L2 defective for SNX17 interaction, or siRNA ablation of SNX17 expression we demonstrate that the interaction between L2 and SNX17 is crucial for viral infection with multiple PV types, indicating an evolutionary highly conserved viral entry mechanism. Loss of the L2-SNX17 interaction dramatically decreases the efficiency with which viral genomes transit to the nucleus. We also show that capsids defective in their capacity to bind SNX17 transit much more rapidly to the lysosomal compartment, suggesting that L2-SNX17 interaction is delaying or redirecting HPV capsids from the major lysosomal pathway. We observed that HPV capsids have a peak of colocalisation with SNX17-positive vesicles 2 hours post-infection, which was confirmed also by viral DNA staining. While vesicular fractioning shows some unspecific relocalisation of SNX17-positive vesicles during an early stage of HPV infection, the duration of stay is strongly HPV L2-dependent. Moreover, partial peri-nuclear localisation of SNX17 later during an HPV-16 infection

suggests that SNX17 is involved also in the transport of the L2-DNA complex to the nucleus. These studies reveal a novel role for SNX17 in aiding viral infection processes, and raise the possibility that normal cellular recycling pathways are perturbed as a result of HPV infection.

P739

Board Number: B1044

Imaging the Alphavirus Exit Pathway.

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Alphaviruses such as Chikungunya virus are important and widely distributed human pathogens, and there are no effective vaccines or antiviral therapies available. Alphaviruses are small, highly organized RNA viruses with a membrane envelope derived by budding through the host cell plasma membrane (PM), excluding host membrane proteins. Viral particles contain an internal nucleocapsid and an external lattice of the viral E2 and E1 transmembrane proteins. While alphavirus entry has been widely studied, the process and dynamics of assembly and budding remain poorly understood. Here, we generated Sindbis viruses (SINV) with fluorescent protein labels on the E2 envelope protein and exploited them to characterize virus assembly and budding in living cells. Using confocal and total internal reflection fluorescence microscopy we observed that during virus infection E2 became enriched in localized patches on the PM and in short filopodia-like extensions. These E2-labeled patches and extensions contained all of the viral structural proteins and the non-structural protein nsP1. Correlative light and electron microscopy studies established that the patches and extensions co-localize with virus budding structures, while light microscopy showed that they exclude a freely diffusing PM marker protein. Protein exclusion required the interaction of the E2 protein with the capsid protein, a critical step in virus budding, and was associated with the immobilization of the envelope proteins on the cell surface. Virus infection induced at least two different types of extensions: the shorter extensions describe above ($3.19 \mu\text{m} \pm 0.94$; $n=30$) that exclude the PM marker, actin and tubulin, and longer extensions ($12.09 \mu\text{m} \pm 0.4$; $n=30$) that contain all of these marker proteins and appear to mediate virus particle transfer. Together our data support a model in which alphavirus budding occurs at specialized sites of virus-induced reorganization of the PM.

P740**Board Number: B1045****Deficient protein palmitoylation in the deadly fungus *Cryptococcus neoformans* affects pathogenesis by altering fungal interactions with host cells.**F.H. Santiago-Tirado¹, M. Yang¹, T.L. Doering¹;¹Molecular Microbiology, Washington University School of Medicine, Saint Louis, MO

Cryptococcus neoformans is the most common fungal cause of meningitis, responsible for over 625,000 deaths every year. Most of these deaths are in immunocompromised patients, historically in the setting of AIDS, but currently also affecting patients with other underlying conditions such as cancer or organ transplant. *C. neoformans* is a facultative intracellular pathogen, and adherence to and uptake by host phagocytes during infection are key events that are central in its pathogenesis. Fungal engulfment by host cells and subsequent intracellular proliferation has been implicated in latency, dissemination, and virulence, but the full complement of *C. neoformans* gene products that participate in these processes has not been defined. To address this question, we used an automated high content imaging method to quantify the interactions between a human macrophage-like cell line and mutant fungi from a partial deletion collection. We identified multiple genes whose deletion led to alterations in the adherence and/or phagocytic indexes. One of the genes identified encode a protein S-acyl transferase, one of a family of DHHC domain-containing proteins that catalyzes lipid modification of proteins. Deletion of this gene, termed *PFA4*, results in enhanced adherence to and phagocytosis by human macrophages. Mutant cells lacking *PFA4* exhibit morphological defects that are exacerbated under host-like conditions, and are sensitive to a variety of cell wall stress conditions *in vitro*. Consistent with these observations, they have a profound defect in intracellular growth and are avirulent in a mouse model of cryptococcal infection. These results suggest a surface defect but, interestingly, the mutant cells show no obvious defect in the polysaccharide capsule that surrounds their cell walls, which is a major virulence factor. They do, however, display altered cell wall structure and increased exposure of surface glycans compared to wild type. Defects in lipid modification may cause mislocalization or degradation of the substrate proteins, leading to their dysfunction. We hypothesize that *C. neoformans* uses palmitoylation to regulate its surface composition and thereby modulate its interactions with the host. Our current efforts are directed at identifying the specific protein substrate(s) responsible for the changes we observe in the *pfa4* mutant. Little is known about the role palmitoylation plays in virulence of this or other pathogenic fungi, hence these findings open a new area of investigation and promises novel avenues for therapeutics based on the role of palmitoylation in fungal pathogenesis.

P741**Board Number: B1046****Comparison of the Role of Human PI 3-kinase Pathway in Internalization of the Bacterial Pathogens *Yersinia enterocolitica* and *Listeria monocytogenes*.**K. Ireton¹, G.C. Dowd¹, R. Thomas¹;¹Microbiology and Immunology, University of Otago, Dunedin, New Zealand

Human type IA Phosphoinositide (PI) 3-kinase plays a critical role in infection by many microbial pathogens, including enteric bacteria (*Listeria monocytogenes*, *Yersinia enterocolitica*, *Campylobacter jejuni*), parasites (*Trypanosoma cruzi* and *Toxoplasma gondii*), and viruses (Ebola, Influenza A virus). PI 3-kinase regulates a multitude of cellular processes by controlling the localization and/or activity of at least 50 downstream proteins. A key unresolved question is how host PI 3-kinase promotes internalization ('entry') of various microbes into human cells. Do different microbes utilize similar or distinct PI 3-kinase-mediated signaling pathways? A previous study employed an RNA interference (RNAi)-based screen to identify nine host downstream effectors of PI 3-kinase needed for entry of the food-borne bacterium *Listeria monocytogenes* (Jiwani et al., 2012. *Infect. Immun.* 3: 1252). The objective of this work was to perform a similar RNAi screen to identify human PI 3-kinase pathway components involved in internalization of *Yersinia enterocolitica*, allowing a comparison of the entry pathways employed by *Listeria* and *Yersinia*. HeLa cells were transfected with an siRNA library targeting ~70 human proteins comprising the type IA PI 3-kinase signaling pathway. Many of the human proteins targeted are known to bind directly to the lipid product of PI 3-kinase, phosphatidylinositol 3,4,5-tris phosphate. The effects of the siRNAs on target gene expression were assessed by real time PCR. siRNAs that inhibited human gene expression were examined for effects on *Yersinia enterocolitica* entry, using a commonly employed antibiotic protection assay. From these studies, we identified eight human proteins in the type IA PI 3-kinase pathway that are needed for internalization of *Yersinia enterocolitica*. These proteins include the GTPase Rab5c, regulators of host Rho or Arf GTPases (Centaurin-alpha1, Dock180, PSCD3), the focal adhesion component FAK, Phospholipase D 2 (PLD2), the scaffolding protein LL5-alpha, and the serine/threonine kinase mTor. At least four of the eight host proteins involved in *Yersinia* entry (Rab5c, Centaurin-alpha1, PSCD3, and mTor) are also needed for *Listeria* uptake. FAK and PLD2 are dispensable for *Listeria* entry. Of the nine PI 3-kinase pathway components involved in *Listeria* uptake, four have no detectable role in *Yersinia* internalization. We conclude that host PI 3-kinase controls entry of *Yersinia* and *Listeria* through both shared and distinct downstream effectors. Future work will focus on the molecular mechanisms by which these downstream effectors promote bacterial infection.

P742**Board Number: B1047****Deciphering the molecular mechanisms of *Listeria* transcytosis across the intestinal epithelium in intestinal organoids.**C. Fevre^{1,2}, N. Barilone^{1,2}, M. Lecuit^{1,2};¹Institut Pasteur, Paris, France, ²Inserm, Paris, France

The human intestinal mucosa is a physical barrier that delineates the frontier between the external environment and the host. It can be crossed by luminal antigens and enteroinvasive microbes. *Listeria monocytogenes* (*Lm*), a Gram-positive foodborne bacterial pathogen responsible for human listeriosis, crosses the intestinal barrier across goblet cells to access the lamina propria and disseminate systemically. This translocation requires the interaction between *Lm* surface protein InlA and its species-specific epithelial receptor E-cadherin, but is independent of LLO (involved in *Lm* escape from the vacuole) and ActA (involved in *Lm* intracellular motility), suggesting that *Lm* is transferred across goblet cells by transcytosis. Beside E-cadherin and microtubules, the host factors involved in *Lm* transcytosis across goblet cells are unknown. *Lm* transcytosis is not observed in *in vitro* cultured epithelial cells, which are either too polarized to express apically accessible E-cadherin, or unpolarized and devoid of *bona fide* apical basal vesicular trafficking. To identify the host factors involved in *Lm* transcytosis across goblet cells, we have used intestinal organoids (miniguts), which recapitulate faithfully *ex vivo* intestinal epithelium differentiation into intestinal epithelial cell subtypes, including goblet cells, and which are genetically amenable. Using high-resolution confocal microscopy to follow the fate of microinjected *Lm* inside minigut lumen, we have shown that, similar to what is observed *in vivo*, *Lm* is translocated through goblet cells, in a microtubule- and InlA-dependent manner but InlB- LLO- and ActA-independent manner, validating the intestinal organoid as a suitable model to identify the host factors involved in this process. Our working hypothesis is that *Lm* takes advantage of E-cadherin recycling from the apical to basolateral pole of enterocytes. In such a scenario, inhibition of E-cadherin recycling would interfere with *Lm* transcytosis. To test this hypothesis, inducible organoid mutants expressing inactive forms of a series of proteins involved in E-cadherin recycling, such as Rab GTPases and exocyst components have been generated. Our preliminary results show that their inhibition prevents *Lm* transcytosis, and experiments are now under way to fully decipher the molecular mechanisms of *Lm* transcytosis across the intestinal epithelium. Intestinal organoids stand as a very promising model for the spatial and temporal studies of the cell biology of host-pathogen interactions, as it preserves cell differentiation, polarization and tissue architecture, which are exploited by pathogens to invade the host.

P743**Board Number: B1048*****Neisseria gonorrhoeae* breaches the epithelial barrier by inducing Ca²⁺ flux and Ca²⁺-dependent activation of non-muscle myosin II for tissue invasion.**L. Wang¹, W. Song², Q. Yu¹, B. Lin¹;¹CBMG, University of Maryland, College Park, MD, ²Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD

Neisseria gonorrhoeae (GC) infects epithelial cells lining the female reproductive tract, which can lead to pelvic inflammatory and disseminated diseases. We have previously shown that GC interactions with epithelial cells induce the transactivation of epidermal growth factor receptor (EGFR); however, how EGFR activation contributes to GC infection is unknown. This study demonstrates that the interactions of GC with the apical surface of polarized epithelial cells induce Ca²⁺ flux and the accumulation of active non-muscle myosin II (NMII) at the apical junction and the apical surface of epithelial cells. GC-induced activation of Ca²⁺ is dependent on EGFR activation, while the activation and redistribution of NMII require Ca²⁺ flux and myosin light chain kinase that is activated by Ca²⁺-bound calmodulin. Inhibition of either GC-induced Ca²⁺ flux or NMII activation blocks GC-induced disruption of the apical junction and reduces GC transmigration, while having no significant effect on GC adherence and invasion into polarized epithelial cells. Compared to wild type GC, a mutated GC strain where all 11 genes of GC surface protein Opa are deleted induces the disassembly of the apical junction and the activation and redistribution of NMII to significantly greater magnitude, consequently transmigrating at a much higher efficacy. Electron microscopic analysis showed that unlike wild type GC, the Opa deletion mutant alters the apical morphology and forms extensive interaction with the apical membrane of polarized epithelial cells. Taken together, our results reveal that GC disrupts the apical junction of the epithelial barrier by inducing EGFR-dependent Ca²⁺ flux and Ca²⁺-dependent activation and reorganization of junction-associated actomyosin, which facilitates GC invasion into the mucosal tissue. These results reveal a new mechanism by which GC establishes infection using signaling and cytoskeletal apparatus of epithelial cells.

P744**Board Number: B1049****Chlamydia psittaci encodes a functional BAR domain Inc protein that targets inverted and everted inclusion membrane extensions and associates with gap junction proteins.**D.A. Phillips¹, R. Hsia², S. Mojica¹, J. Ravel³, P. Bavoil⁴;¹Department of Microbial Pathogenesis, University of Maryland, Baltimore, Baltimore, MD, ²Core Imaging Facility, University of Maryland, Baltimore, Baltimore, MD, ³Institute for Genome Sciences, University of Maryland, Baltimore, Baltimore, MD, ⁴Department of Microbial Pathogenesis, University of Maryland School of Dentistry, Baltimore, MD

Bacteria of the genus *Chlamydia* are ubiquitous pathogens that grow strictly within an intracellular plasma membrane-derived vacuole, termed the chlamydial inclusion. *Chlamydia trachomatis* (Ctr), a prevalent sexually transmitted pathogen, causes infection that is usually limited to the human genital or ocular mucosal epithelia. In contrast, *Chlamydia psittaci* (Cps), a prevalent avian pathogen, is an aggressive, tissue-invasive pathogen that can cause life-threatening zoonotic infection in humans and animals. We have proposed that chlamydiae of all species replicate while in contact with the chlamydial inclusion membrane (CIM). Chlamydiae secrete type III secretion (T3S) effector proteins into and across the CIM, whose roles are to subvert host activities to benefit chlamydial growth. Among these are proteins of the Inc family characterized by a bilobar hydrophobic sequence serving to anchor Incs into the CIM. The IncA ortholog of Cps differs from IncAs in other *Chlamydia* spp. owing to the presence of a predicted BAR domain within its C-terminus closely related to BAR domains found in eukaryotic sorting nexins. Purified recombinant IncA (IncA/Cps) was able to reconfigure liposomes into tubules *in vitro*, a canonical test for BAR domain functionality. In contrast, neither IncA/Ctr nor IncA/Cps with a BAR R188G mutation produced tubules. Transfected HEK293T cells expressing GFP-IncA/Cps displayed fluorescent tubular/fibrous structures in the cell cytosol. Cps-infected HeLa cells stained with anti-IncA/Cps antibodies revealed similar inverted structures within the lumen of the inclusion that did not co-localize with replicating chlamydiae, as well as everted CIM extensions into the cytosol. The former but not the latter were ablated when infected cells were exposed to the sphingolipid biosynthesis inhibitor myriocin, suggesting that the IncA-positive inclusion lumen structures are continuous with the inclusion membrane. Further, transfected IncA/Cps fused to the promiscuous biotin ligase BirA resulted in the specific biotinylation (Bio-ID) of several proteins of tight junctions and vesicular trafficking. In summary, our study demonstrates that *C. psittaci* expresses and secretes an Inc protein with a functional BAR domain that is absent in orthologs of other *Chlamydia* spp. While this the first characterized prokaryotic BAR domain, it is consistent with the need of intracellular chlamydiae to acquire eukaryotic-like functions to subvert host cell function. IncA-positive inverted extensions are consistent with the need of Cps to replicate in contact with the CIM. Everted extensions and the identification of tight junctions and vesicular trafficking proteins as targets of IncA suggest a possible role in intercellular trafficking.

P745**Board Number: B1050****Analysis of the interaction and internalization of *Bacillus thuringiensis* Cry toxins in different human cell lines.**R.G. Silva-Delgado¹, K. Leyva², B.E. Garcia-Perez², J.M. Favela-Hernandez³, G. Guerrero⁴;¹Facultad de Quimica-UJED, Gomez Palacio, Dgo, Mexico, ²Immunologia, Escuela Nacional de Ciencias Biologicas IPN, Mexico City, Mexico, ³Facultad de Quimica-UJED, Gomez Palacio, Mexico, ⁴Unidad Academicas de Ciencias Biologicas-UAZ, Zacatecas, Mexico

Bacterial toxins have the ability to mount significant immune responses at the systemic and mucosal sites. *Bacillus thuringiensis* Cry toxins has been used for decades in the biological control of insects (López et al., 2013 Review). However, several studies *in vivo* and *in vitro* support its role as strong immunogens (Vazquez-Padrón et al., 1999; 2000; Moreno-Fierros et al., 2000; Guerrero et al., 2004), adjuvants (against *Naegleria fowleri* ameba) (Rojas-Hernández et al., 2004:2010) and carriers of clinically important antigens (Moreno-Fierros et al., 2003; Guerrero et al., 2007ab ; Esquivel R and Moreno-Fierros L., 2005). Furthermore, it has been showed that they induce the expression of the pIgR(Reséndiz-Albor et al., 2010) as well as the FcRn(Verdín-Terán et al., 2009) in the small intestine. Despite of these wealth of data, remains to be defined and clarified the effects of the interaction and internalization of the Cry toxins with epithelial cells from the gut and lung of mammals. The aim of the present work is to analyze the interaction and internalization of the Cry toxins using different human cell lines. A kinetic of internalization using immunofluorescence analysis was followed. At very early times, particles labelled+ Cry toxin-10 µg/ml started the interaction with proteins-like-receptors in the surface of the A549 cell lines, and THP-1 respectively (macrophages derived with PHA) and at later times, particles labelled + Cry toxin internalized by process called macropinocytosis. These data would implicate that once ingested (by oral route) or inhaled by intranasal route), *Bt* Cry toxins are internalized by the different cell in the gut or lung of the mammals and trigger responses that are key for the observed immunogenic and adjuvant properties in mice.

P746**Board Number: B1051****pH Alkalinization by Chloroquine Suppresses *Burkholderia* T6SS-1 Gene Expression, Phagosomal Escape and Multi-Nucleated Giant Cell Formation.**J. Chua¹, J. Senft¹, S. Lockett², P. Brett³, M. Burtnick³, D. Deshazer¹, A. Friedlander¹;¹Bacteriology, USAMRIID, Fort Detrick, MD, ²Optical Microscopy and Analysis Laboratory, Frederick National Laboratory for Cancer Research, Frederick, MD, ³Microbiology and Immunology, University of South Alabama, Mobile, AL

Burkholderia mallei and *B. pseudomallei*, Gram negative facultative intracellular pathogens, cause the often fatal diseases glanders and melioidosis, respectively, in humans and animals. A morphological

hallmark of pathogenesis is formation of granulomas containing multi-nucleated giant cells (MNGCs) and necrosis in different organs. These processes are dependent on the Type VI secretion system-1 (T6SS-1), which is required for virulence in animals and regulated by the two component regulatory system VirAG. To understand the disease mechanisms and develop treatments, we examined the cell biology of MNGC formation and cell death. Chloroquine (CLQ) inhibits these processes by retarding intracellular growth and preventing phagosomal escape of the pathogens. This depends on the ability of CLQ to neutralize acid pH intracellularly because other alkalinizing compounds also inhibit phagosomal escape and MNGC formation. By alkalinizing the endocytic pathway, CLQ hampers bacterial virulence protein expression because T3SS and T6SS/ VirAG regulated protein expression is induced at acid pH. Specifically, acid pH upregulates the expression of a Type VI secretory apparatus protein Hcp1 and the deubiquitinase TssM. CLQ treatment of *Burkholderia* infected Madagascar Hissing Cockroaches increases survival. This study highlights multiple mechanisms by which CLQ, through alkalinization of phagosomal pH, inhibits growth and virulence and argues for CLQ's inclusion in treatment for *Burkholderia* diseases.

P747

Board Number: B1052

Junin virus infection triggers the autophagy pathway in a human cell line.

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Autophagy is a conserved degradative pathway that plays a key role in maintaining cellular homeostasis through eliminating unwanted proteins and damaged organelles by cellular self-digestion in the lysosome to fulfil the demand for aminoacids required for cell survival. The first step of this process is the formation of double membrane vesicles, denominated autophagosomes, around portions of cytoplasm which then fuse with lysosomes where enzymatic degradation occurs. This process has a crucial role in protecting cells from viral invasion. However, viruses have been able to subvert this pathway in their own benefit. Junin virus (JUNV) belongs to the phylogenetic clade B of the Arenaviridae family. It is the aetiological agent of Argentine haemorrhagic fever, an endemo-epidemic disease affecting the population of the most fertile farming land of Argentina. We analysed the role of autophagy in the course of JUNV infection employing human cell line A549. We used LC3 as a protein marker of autophagy pathway modulation after viral infection. Mammalian LC3 is a cytoplasmic protein (LC3-I) that becomes lipidated and membrane associated (LC3-II) upon induction of autophagy. When detected by confocal laser scanning microscopy, vesicles associated LC3-II appears as discrete green dots. We have observed that cells overexpressing EGFP-LC3 and infected with JUNV showed an increased number of LC3 dots similar to what we have shown after starvation- or BafilomycinA1-treatment, which leads to autophagosome formation induction or accumulation, respectively. We have monitored the conversion of LC3-I (cytosolic) to LC3-II (associated to autophagosomes) by Western blot technique observing that levels of LC3-II in JUNV-infected cells were similar to that observed in starved cells. Moreover, cells pre-treated with rapamycin, a pharmacological autophagy inductor, enhanced

virus yield with respect to the control situation. In addition, we have assayed the replication capacity of JUNV in Atg5 knock-out cells (a key molecular component of the autophagic pathway), but no differences were found when compared to the wild type Atg5 cells. At last, we have kinetically studied the number of LC3 dots after JUNV infection over a period of 24 h post infection (p.i.) and found that the pathway was activated by the presence of the virus since 2 h p.i. and remained activated until the end of the mentioned lapse of timer monitored. All together, these results allowed us to conclude that JUNV infection leads to an autophagic response in the infected cells. However, a functional autophagy pathway does not seem to be required for efficient virus replication.

P748

Board Number: B1053

Characterization of the vesicular donor compartments to Yersinia-containing vacuoles positive for LC3 and with a single limiting membrane.

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Yersinia pseudotuberculosis is an enteropathogenic bacterium which is able to replicate within both macrophages and epithelial cells by subverting the autophagy pathway. The autophagy is characterized in its classical form by the formation of vacuoles with double membrane and displaying LC3 proteins giving rise to a specific internal vesicles so-called: autophagosomes. We showed that in function of cells types *Y. pseudotuberculosis* is contained in two different LC3 positive vacuoles. In fact, in macrophages the bacteria could block the autophagosome maturation, to create a replicative niche with double limiting membranes (Moreau et al. 2010). While, in the epithelial cells, *Y. pseudotuberculosis* can replicate inside non-acidic vacuoles displaying the autophagic marker, LC3, but with only one limiting membrane (Ligeon et al. 2014). The autophagosome formation during bacteria invasion is characterized by the dynamic rearrangement of cell membrane structures via multiple membrane fusion events (Ligeon et al., 2011). Membranes forming the autophagosome can result from fusion of different vesicular donor compartments (ER, mitochondria, endosomes,...). Here, we investigated which donor compartment plays a role during the formation of the Yersinia-containing vacuoles (YCVs). In epithelial cells, we observed fusion of YCVs with different types of endosomes vesicles and LC3-positive small vesicles. We propose that both the autophagy and the endocytic pathway participate as membrane sources for the growing YCV during bacteria entry and replication.

Moreau et al. Cellular Microbiology (2010) 12:1108-1123 Ligeon et al. Cellular Microbiology (2011) 13:1303-1311 Ligeon et al. Autophagy (2014) in press

P749**Board Number: B1054****Activation of Focal Adhesion Kinase by Salmonella Suppresses Autophagy via an Akt/mTOR Signaling Pathway and Promotes Bacterial Survival in Macrophages.**K.A. Owen¹, C.B. Meyer¹, A.H. Bouton², J. Casanova¹;¹Cell Biology, University of Virginia, Charlottesville, VA, ²Microbiology, University of Virginia, Charlottesville, VA

Autophagy has emerged as an important antimicrobial host defense mechanism that not only orchestrates the systemic immune response, but also functions in a cell autonomous manner to directly eliminate invading pathogens. Pathogenic bacteria such as *Salmonella* have evolved adaptations to protect themselves from autophagic elimination. Here we show that signaling through the non-receptor tyrosine kinase focal adhesion kinase (FAK) is actively manipulated by the *Salmonella* SPI-2 system in macrophages to promote intracellular survival. In wild-type macrophages, FAK is recruited to the surface of the Salmonella-containing vacuole (SCV), leading to amplified signaling through the Akt-mTOR axis and inhibition of the autophagic response. In FAK-deficient macrophages, Akt/mTOR signaling is attenuated and autophagic capture of intracellular bacteria is enhanced, resulting in reduced bacterial survival. We further demonstrate that enhanced autophagy in FAK^{-/-} macrophages requires the activity of Atg5 and ULK1 in a process that is distinct from LC3-assisted phagocytosis (LAP). *In vivo*, selective knockout of FAK in macrophages resulted in more rapid clearance of bacteria from tissues after oral infection with *S. typhimurium*. Clearance was correlated with reduced infiltration of inflammatory cell types into infected tissues and reduced tissue damage. Together, these data demonstrate that FAK is specifically targeted by *S. typhimurium* as a novel means of suppressing autophagy in macrophages, thereby enhancing their intracellular survival.

Immune System**P751****Board Number: B1057****TNC Induced Regulation of IL-12 Promoted Apoptosis among DP Thymocytes.**S. Miller¹, S. Henley¹, F.R. Davis¹, L. Johnson², M. Martinez³;¹Integrative Biosciences, Tuskegee University, Tuskegee, AL, ²Biology, Morgan State University, Baltimore, MD, ³Biology, Tuskegee University, Tuskegee, AL

Central tolerance of thymocytes is a dynamic process involving complex interactions between cytokines, developing thymocytes and the cells of the thymic stroma. Among the cells of the thymic stroma are thymic nurse cells (TNCs). TNCs are cortical epithelial cells of the thymus that appear to play a significant role in central tolerance of T-cells. TNCs specifically interact with and internalize CD4⁺CD8⁺TCR^{lo} double positive (DP) thymocytes. Thymocytes at this developmental stage are

undergoing MHC restriction, a process where the cells learn to recognize self MHCs. DP thymocytes are reported to be non-responsive to a variety of cytokines, yet responsive to IL-12. IL-12 is a Th1 type cytokine whose secretion is upregulated following interactions between CD40 on APCs and CD40 ligand (CD154) on T-cells. Although IL-12 promotes pro-inflammatory responses in the periphery, it influences the deletion of the DP thymocytes in the thymus. Prior work in this lab has demonstrated that IL-12 secretion is significantly increased in co-cultures containing TNCs where negative selection is high. However, it could not be determined whether the TNCs themselves were the source of the increased IL-12 concentrations. Therefore, we hypothesize that TNCs can secrete IL-12 via CD40/CD40L interaction, and that IL-12 plays a role in the interaction between TNCs and DP thymocytes. Using in vitro experimental systems composed primarily of TNC cultures or TNC/thymocyte co-cultures, we investigated the potential for TNCs to secrete IL-12 in co-cultures and the effects of IL-12 on thymocytes in TNC/thymocyte co-cultures. Lipopolysaccharide stimulated TNCs demonstrated time dependent increases IL-12 secretion and correlative increases in CD40 expression. Furthermore, thymocytes cultured with TNCs also demonstrated a time dependent increase in CD154, regardless of negative selection conditions.

However, thymocyte apoptosis was found to be severely reduced among DP thymocytes in all co-cultures containing TNCs. And thymocyte rescue from apoptosis observed in cultures that included recombinant IL-12 was found to be greater than observed in cultures which did not include the cytokine. Reductions in apoptosis correlated with increases in thymocyte maturation and significant reduction of the IL-12 receptor on DP thymocytes in these co-cultures. These results lead us to conclude that TNCs can regulate DP thymocyte numbers by both increasing apoptosis through IL-12 secretion and decreasing apoptosis by downregulating the IL-12 receptor.

P752

Board Number: B1058

The role of interleukin-27 receptor signaling in the development of abdominal aortic aneurysm.

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Rationale: Abdominal aortic aneurysm (AAA) is a cardiovascular disease, which is characterized by aortic wall dilation with subsequent rupture and internal bleeding. Inflammation in the vessel wall is a key component of atherosclerosis development and could also contribute to AAA formation. Various pro-inflammatory cytokines were investigated in atherosclerosis and AAA, while little is known about the contribution of anti-inflammatory cytokines with regard to their ability to control vascular inflammation. Interleukin (IL)-27 is a recently discovered member of the IL-6/IL-12 family. The IL-27 receptor is composed of IL-27 receptor A (WSX-1) and gp130 and is required for all established IL-27 signaling pathways. The functions of IL-27 and its receptor signaling in AAA remain unknown. Objective: The aim of this study was to test whether IL-27R signaling is required to suppress abdominal aortic aneurysm

formation in Angiotensin II mouse model. Methods and Results: Angiotensin II containing pumps were surgically implanted into Il27ra^{-/-} x Ldlr^{-/-} and Il27ra^{+/-} x Ldlr^{-/-} control mice with already developed atherosclerosis. The progression of AAA was analyzed 4 weeks later. We found that Il27ra^{-/-} x Ldlr^{-/-} mice developed large AAA with visual hemorrhage into artery wall compared to Il27ra^{+/-} x Ldlr^{-/-} counterparts. Augmented disease correlated with increased accumulation of myeloid cells and T cells in the area of AAA formation. Preliminary analysis of gene expression profile revealed upregulation of IFN γ , TNF, CCL5, S100A9, MMP9 - possible players in pathogenesis of AAA. Conclusions: Altogether our data suggest that IL-27R signaling may play an important role in suppression of inflammation in aortic wall during AAA development.

P753

Board Number: B1059

CELL-SPECIFIC APPROACHES TO DISSECTING PUTATIVELY FUNCTIONAL GENETIC ASSOCIATIONS IN PEDIATRIC AUTOIMMUNE DISEASES.

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Autoimmune (AI) disorders affect 1 in 12 people living in the Western Hemisphere, representing a significant cause of chronic morbidity and disability. As demonstrated by the identification of hundreds of susceptibility genes by genome wide association studies (**GWAS**), genetic risk factors play an important role. These results show that many loci are shared across clinically-distinct disease groups, consistent with AI diseases having common etiologies.

To investigate the genetic architecture of pediatric autoimmune diseases (**pAIDs**), we performed a heterogeneity-sensitive GWAS (**hsGWAS**) across 10 pAIDs in a nested case-control study including over **6,000** cases and nearly **11,000** controls. We identified **137** independent loci nominally associated with one or more pAIDs, **31** of which show evidence of being pan-pAID significant ($P_{PPS} < 5 \times 10^{-8}$), when adjusted for the use of a common control. A number of the top SNPs map to or near candidate genes with established or compelling immunoregulatory functions (e.g., **CD40LG**: $P_{PPS} < 5.37 \times 10^{-9}$ and **TNFSF11B**: $P_{PPS} < 8.43 \times 10^{-10}$). All **31 PPS** lead SNPs, or their nearby proxies ($r^2 > 0.8$ within 1MB), are known coding ($n=4$), regulatory ($n=22$), conserved ($n=19$) or literature-supported ($n=27$) SNPs. The lead loci are specifically enriched for DNase hypersensitivity sites ($p < 0.01$), eQTLs, and coding variants ($p < 0.001$, respectively). To examine if there is evidence for cell-type specific functional

enrichment, we annotated pAID-associated variants using Encode transcription factor binding (TFBS), histone modification (HM), digital genomic footprinting (DGF), and DNase hypersensitivity (DH) datasets across available cell types. Compared to the genomic background, the 137 lead associated SNPs ($P_{\text{nominal}} < 1 \times 10^{-6}$) are consistently enriched for regulatory functions across Monocytes, Th1, Th2, Treg, and lymphoblastoid B-cells (Bonferroni-adjusted $P_{\text{adj}} <$ from 3×10^{-7} to $P_{\text{adj}} < 0.047$). We further characterized the expression profiles of candidate genes across over three hundred unique human and murine tissues and immune-specific cell types. We show the expression of candidate pAID-associated gene transcripts are enriched across immune cells and we have identified sets of genes shared by two or more related diseases with closely correlated expression profiles, for example Type-1-Diabetes and Thyroiditis. Integration of multiple *in silico* analytical approaches has identified highly-shared autoimmune signals (e.g., ***IL2RA*: $P_{\text{PPS}} < 2.41 \times 10^{-10}$**) associated with 6 different pAIDs and demonstrating converging roles for the JAK-STAT, innate, and TH₁-TH₂/TH₁₇ mediated T-cell signaling molecular pathways, which are attractive pharmacological targets for future functional studies.

P754

Board Number: B1060

Substance P enhances mesenchymal stem cells-mediated immune modulation.

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Since clinical application of MSCs requires long-term ex vivo culture inducing senescence in MSCs and reducing the therapeutic activity of transplanted MSCs, numerous efforts have been attempted to sustain the active state of MSCs. Substance P (SP) is a neuropeptide that functions to activate the cellular physiological responses of MSCs, including proliferation, migration, and secretion of specific cytokines. In this study, we explored the potential of SP to restore the weakened immune modulating activity of MSCs resulting from long-term culture by measuring T cell activity and interleukin-2 (IL-2) secretion of CD4+ Jurkat leukemic T cells. As the number of cell passages increased, the immunosuppressive function of MSCs based on T cell activity decreased. This weakened activity of MSCs could be restored by SP treatment and nullified by co-treatment of an NK1 receptor blocker. Higher levels of transforming growth factor beta 1 (TGF-beta1) secretion were noted in the medium of SP-treated late passage MSC cultures, but IL-10 levels did not change. SP-treated MSC-conditioned medium decreased T cell activity and IL-2 secretion in CD4+ Jurkat leukemic T cells even in the presence of lipopolysaccharide (LPS), both of which were successfully blocked by inhibiting the TGF beta signaling pathway. This stimulatory effect of SP on late passage MSCs was also confirmed in direct cell-cell contact co-culture of MSCs and T cells. Collectively, our study suggests that SP pretreatment to MSCs may recover the immunosuppressive function of late passage MSCs by potentiating their ability to secrete TGF-beta1, which can enhance the therapeutic activity of ex vivo expanded MSCs in long-term culture. Keywords: Substance P, mesenchymal stem cells, T cells, TGF-beta1, IL-2, immune modulation.

P755**Board Number: B1061****Substance P ameliorates collagen II-induced arthritis in mice via suppression of the inflammatory response.**P. Ju Hyung¹, H. Hong², Y. Son^{3,4};¹Graduate School of Biotechnology, Kyung Hee University, Yong-In, Korea, ²College of Medicine, Kyung Hee University, Seoul, Korea, ³Graduate School of Biotechnology, Kyung Hee University, Yongin, Korea, ⁴Department of Genetic Engineering, Kyung Hee University, Yongin, Korea

Rheumatoid Arthritis (RA) is a chronic autoimmune disease with systemic inflammatory disorder that may mainly attack synovial joints. Main therapy for RA has been focused in the suppression of inflammation to alleviate symptoms. Recently, substance-P (SP) was found to have functions as an injury-inducible endogenous factor to mobilize bone marrow stem cell to wound site and anti-inflammatory agent. Considering the function of SP, it was expected that SP may exerted the therapeutic effect on RA. In this study, SP effect was explored using collagen type 2-induced arthritis (CIA) animal model that was induced by injecting emulsion containing 100µg of collagen II (CII) into subcutaneous tissue of the tails and 3 weeks later, boosting with 100 µg CII emulsified with an equal volume of incomplete Freund's adjuvant. At 4 weeks post immunization, the mouse showed CIA phenotype and SP was injected twice a week to observe phenomenon of RA. In result, compared to vehicle-treated group, SP reduced local inflammatory signs such as swelling and erythema of the paw and inhibited joint cartilage degradation. Moreover, SP treatment reduced the size of spleens which became enlarged due to excessive inflammation, increased IL-10, anti-inflammatory cytokine and decreased TNF-alpha and IL-17, pro-inflammatory cytokines in the serum, indicating that SP inhibited systemic inflammation. Our study revealed that SP might have a beneficial effect for RA, which may be occurred by inhibition of the inflammatory responses. Furthermore, SP is anticipated to be utilized to treat diverse autoimmune diseases as well as RA.

P756**Board Number: B1062****IMMUNE RESPONSE TO BACTERIAL LYSATES MIXTURE INJECTION IN A RAT MODEL.**D. Cid¹, I. Vargas¹, S. Sanchez², R.M. Ramirez³, M.A. Lopez¹, D.A. Garcia^{1,2}, E.E. Lopez¹;¹Lab. Biología Celular y Neurobiología, Univ Autónoma De Zacatecas, Zacatecas, Mexico, ²Univ Autónoma De Zacate, Guadalupe, Mexico, ³Lab. de Enfermedades Infecciosas, Universidad Autónoma de Zacatecas, Zacatecas, Mexico

BACKGROUND: The bacterial lysates mixtures are widely used in patients with repeated respiratory infections. **METHODS:** We took three study groups, each with three rats. The Group 1, Study Group,

received the injection of a Bacterial Lysate Mixture (Staphylococcus aureus 300mmol/ml, Staphylococcus albus 300mmol/ml, non-hemolytic Streptococcus 100mmol/ml, Neisseria catarrhalis 100mmol/ml and Candida albicans 100mmol/ml). Group 2, positive control, received Lipopolysaccharide (LPS) (e. coli strain O111:B4). To group 3, negative control, was administered Evans solution. The way of application was subcutaneous injection in the spine of the rat. All groups were injected with increased doses at day 0, and day 8, with taking samples of blood at time zero, 24 hours, day 8, and day 15. Blood from the eye corner was obtained for measurement of cytokines IL-1b, IL-4, IL-10 and IFN-g by ELISA assay, and complement C3 and IgG antibody by radial immunodiffusion assay. RESULTS: In the case of Group 1 with bacterial lysates mixture (BLM), there is an initial response to 24 hrs with a decrease cytokines with respect to its basal level and a final increase at fifteenth day. In the statistical analysis there is a difference of concentrations of IL-10 in the 24hrs sample in group of bacterial lysates mixture (24.7pg/ml) compared to LPS group (117.9pg/ml) with a p value = 0.049. The levels of IL-1b shows a significant difference at 24 hours between the groups BLM and LPS (63.1 pg/ml vs 92.6pg/ml, respectively); p value = 0.047; and a significant difference in IL-4 between BLM and LPS, in this case at 8 days (52.9 pg/ml vs 0.00 pg/ml) p value = 0.024. The difference of groups in INF-g was significant in the measurement at 15 days, in this case BLM vs LPS (24.2. pg/ml vs 52.4 pg/ml, respectively, p value = 0.030) and LPS vs Evans solution (52.4 pg/ml vs 23.9 pg/ml, respectively, p value = 0.028). In the case of complement C3 fraction, there was a difference between BLM and LPS at 24hrs (2000.33 mg/dl vs 2422.33 mg/dl, respectively, p value = 0.045). IgG antibody measure there was no difference in the three groups. CONCLUSIONS: Our results show a change in immune response to stimulus with Bacterial Lysates Mixture in this rat model, identifying an effect at 24hrs for IL-10, IL-1b and C3 complement, and at 8th day for IL-4, between BLM and LPS groups, suggesting the greater effect of Bacterial Lysates is short term.

P757

Board Number: B1063

Substance P enhances M2 phenotype of resting microglia and induces skewing from interferon- γ and tumor necrosis factor- α -stimulated M1 toward M2 microglia.

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Microglia are resident immune cells of central nervous system (CNS). In healthy brain, microglia are resting status and regulate brain homeostasis. Microglia become activated after brain injury either classically activated or alternatively activated referred as M1 and M2 type, respectively. It has been known that pro-inflammatory M1 type shows detrimental roles in injured environment, whereas anti-inflammatory M2 type has neuroprotective properties actively involved in tissue repair. We firstly identified protective roles of substance P (SP) in tissue repair from CNS injury by induction of M2 macrophages and microglia. In this study, we aimed to determine the effects of SP in primary cultured microglia. To obtain microglia, mixed glial cell culture was prepared from brain of postnatal day 1-3 inbred Lewis rat. Microglia were isolated by magnetic-activated cell sorting using CD11b antibody.

Primary cultured microglia showed mixed phenotypes of amoeboid, intermediate, and ramified morphology and considered as resting microglia. Characterization of microglia was confirmed by immunofluorescence staining using microglia specific antibodies against CD11b, ionized calcium-binding adapter molecule 1 (Iba-1), and CD68. Resting microglia showed anti-inflammatory M2 phenotype determined by high expression of CD206, CD163, and CX3CR1 with phagocytic activity of *E.coli* particles. Administration of SP significantly increased CD163 and CX3CR1 expression, suggesting enhanced M2 phenotype of resting microglia. To further analyze SP-induced M2 polarization from M1 type, we stimulated resting microglia by tumor necrosis factor- α (TNF α) and interferon- γ (IFN γ), mimicking injury-induced inflammation, with or without SP. In TNF α and IFN γ -stimulated microglia, CX3CR1 was significantly reduced, suggesting M1 microglia. However, administration of TNF α and IFN γ with SP completely nullified reduction of CX3CR1, moreover, increased expression of CX3CR1 was detected. Taken together, SP contributes to polarization of M2 microglia from resting and M1-activated phenotype.

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P758

Board Number: B1064

PPAR γ activation following apoptotic cell instillation promotes resolution of lung inflammation and fibrosis.

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Changes in macrophage phenotype have been implicated in apoptotic cell-mediated immune modulation via induction of peroxisome proliferator-activated receptor gamma (PPAR γ). In this study, we characterized PPAR γ induction by apoptotic cell instillation over the course of bleomycin-induced lung injury mice. Next, the role of PPAR γ activation in resolving lung inflammation and fibrosis was investigated. Our data demonstrate that apoptotic cell instillation after bleomycin results in immediate and prolonged enhancement of PPAR γ mRNA and protein in alveolar macrophages and lung. Moreover, PPAR γ activity and expression of its target molecules, including CD36, macrophage mannose receptor, and arginase 1, were persistently enhanced following apoptotic cell instillation. Co-administration of the PPAR γ antagonist, GW9662, reversed the enhanced efferocytosis, and the reduced pro-inflammatory cytokine expression, neutrophil recruitment, myeloperoxidase (MPO) activity, and hydroxyproline contents in the lung by apoptotic cell instillation. In addition, inhibition of PPAR γ activity reversed the expression of transforming growth factor beta (TGF- β), interleukin (IL)-10, and hepatocyte growth factor (HGF). These findings indicate that one-time apoptotic cell instillation contributes to anti-inflammatory and anti-fibrotic responses via upregulation of PPAR γ expression and subsequent activation, leading to regulation of efferocytosis and production of pro-resolving cytokines.

P759**Board Number: B1065****Itch Agonists as a Novel Approach to Regulate Immune Responses in Asthma.**S. Agarwal¹, M. Eddins¹, M. Kodrasov¹, J.G. Marblestone¹, J. Wu¹, J. LaRocque¹, M.R. Mattern¹, P. Oliver², S. Kumar¹;¹RandD, Progenra Inc., Malvern, PA, ²Children's Hospital of Philadelphia, Philadelphia, PA

Asthma is a chronic disease with considerable morbidity and no cures. Current treatments are symptomatic and although efficacious, can produce significant adverse effects. Thus, improved, targeted therapies for asthma are needed. The immune response leading to the development of asthma involves differentiation of T cells (CD4+) to effector T cells, which secrete cytokines that assist in the recruitment and proliferation of various innate immune cells. TH2 and TH17 cells contribute to pathogenesis by secreting inflammatory cytokines that induce the influx of neutrophils and eosinophils, leading to airway inflammation. Targeted suppression of pro-inflammatory cytokines and chemokines is thus an attractive strategy to dampen allergic responses in diseases such as asthma. Our approach exploits the ubiquitin proteasome pathway to identify a novel therapeutic. Protein ubiquitylation is a key regulatory mechanism of innate and adaptive immune systems. The E3 ligase Itch plays a significant role, negatively regulating the allergic immune response in asthma. Notably, genetic disruption of Itch in mice or humans has been shown to cause multi-system immune disorders and lung inflammation. Itch belongs to a family of E3 ligases that exist in an auto-inhibited state; it is kept inactive by the N-terminal auto-inhibitory domain and is activated by an adapter protein, Ndfip1, which relieves the auto-inhibition. Ndfip1 promotes Itch activity and, in doing so, limits TH2 differentiation and cytokine production in T cells, as well as the pathogenicity of TH17 cells. Significantly, Ndfip1^{-/-} mice develop inflammation in the lungs characteristic of that induced by TH2 cytokine producing T cells. Thus, Ndfip1 is a key negative modulator of T cell regulation and allergic inflammatory responses. A therapeutic mimetic of Ndfip1 should diminish allergic inflammation by selectively activating Itch mediated ubiquitylation, thereby limiting TH2 and TH17 cytokine production. Here we report that Progenra has developed and adapted their proprietary E3 ligase assay technology to identify activators of Itch to promote ubiquitylation. The most promising compounds are being analyzed in TH2 cells for proof of concept in assays measuring relevant allergy responses. Data will be presented summarizing our progress to date in targeting Itch for regulating the immune response in asthma.

P760**Board Number: B1066****Intrathymic Transplants of Thymic Nurse Cells Reduced Onset of Lupus-Like Symptoms in NZBWF1 mice.**S. Henley¹, S. Miller¹, F.R. Davis¹, M. Martinez², T. Graham³;¹Integrative Biosciences, Tuskegee University, Tuskegee, AL, ²Biology, Tuskegee University, Tuskegee, AL,³School of Veterinary Medicine, Tuskegee University, Tuskegee, AL

Thymic nurse cells (TNCs) are cortical epithelial cells which interact only with TCR^{lo}CD4⁺CD8⁺ (triple positive or TP) thymocytes. TNCs, which express class I and class II major histocompatibility (MHC) proteins, autoimmune regulator (AIRE) and T restrictive antigens (TRA), play a significant role in MHC restriction. TNCs are significantly reduced in all mouse models of systemic lupus erythematosus (SLE) with disease onset and progression. We hypothesized that defective TNC function in SLE mice contributes to reduced thymocyte apoptosis and in turn the release of potentially autoreactive T cells from the thymus. To investigate this normal TNC lines were created from BALB/cJ mice and transferred into the thymi of haplotype-matched 12 week old lupus prone NZBWF1 female mice. Serum, collected biweekly from recipient mice, was monitored by ELISA for anti-dsDNA antibodies and pro-inflammatory cytokines. Recipient NZBWF1 mice were humanely sacrificed 20 weeks post transplantation and kidneys were harvested for histological analyses. Urine collected at time of sacrifice was analyzed for total protein content. We observed that circulating levels of anti-DNA IgGs were consistently lower in animals treated with TNCs than in SHAM-treated control animals. Proteinuria was also significantly less in TNC-treated animals than in SHAM treated animals and immune complex glomerulonephritis was not observed in the kidneys of TNC-treated animals. These results suggest that intrathymic transplants of normal TNCs reduced SLE disease onset in NZBWF1 mice.

P761**Board Number: B1067****The study of ER stress regulator, Bi-1 protection against by Acetaminophen.**G. Lee¹, H. Kim², H. Chae^{1,3};¹Department of Pharmacology , Chonbuk National University medical school, Jeonju, Korea,²Department of Dental Pharmacology, Wonkwang University, Iksan, Korea, ³Department of Pharmacology , Chonbuk National University medical school, Jeonju, Korea

The liver is a major site of drug metabolism and elimination and as such is susceptible to drug toxicity. Drug induced liver injury is a leading cause of acute liver injury, of which acetaminophen (APAP) is the most frequent causative agent. APAP toxicity is initiated by its toxic metabolite NAPQI. However, downstream mechanisms underlying APAP induced cell death are still unclear. Endoplasmic reticulum (ER) stress and the unfolded protein response (UPR) have recently emerged as major regulators of metabolic homeostasis. The results show that intraluminal redox imbalance of the ER and consequential activation of signaling processes and proapoptotic events are involved in hepatocellular damage caused

by AAP overdose. Our data demonstrate tight connections between ER and cytosol to guarantee redox exchange across compartments: a reducing cytosol is important to ensure disulfide isomerization in secretory proteins. BI-1 were identified that Nrf-2 dependent regeneration of glutathione by GSR, GCL and GS plays a critical role in protection against APAP induced oxidative stress. ER stress regulator, BI-1 knock-out condition promotes cell death. We analyzed the role of UPR and BI-1 in mediating APAP hepatotoxicity. A toxic dose of APAP was orally administered to wild type (wt) and BI-1 knockout (KO) mice and damage mechanisms were assessed. BI-1 KO mice were damaged from APAP and exhibited increased liver necrosis and. APAP metabolism in BI-1 KO mice was disturbed and glutathione was more depleted compared with wt. In BI-1 knock-out mice, ER stress and UPR activation were more susceptible following APAP administration. We suggest that the arguments for and against a role for glutathione in facilitating disulphide-bond formation and consider its role in protecting the cell from endoplasmic-reticulum-generated oxidative stress. Thus, BI-1 plays a anti-damage role in response to APAP intoxication

P762

Board Number: B1068

The Involvement of Mesotocin in Incubation Behavior in the Female Native Thai Chicken.

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Oxytocin (OT) is well known to induce and regulate maternal behaviors in mammals via the supraoptic nucleus and paraventricular nucleus (PVN). In the native Thai chicken, mesotocin (MT; the avian homolog of OT) is associated with the reproductive cycle and rearing behavior in the nucleus preopticus medialis (POM) and PVN, whereas the function(s) of MT in incubation behavior is poorly understood. Therefore, the aim of this study was to investigate the role of MTergic system on incubation behavior in the native Thai chicken. Changes in the number of MT-immunoreactive (-ir) neurons that determined by immunohistochemistry in the nucleus supraopticus, pars ventralis (SOv), POM, and PVN of incubating hens (INC) were compared with those of nest-deprived hens (ND) on days 6, 10, 18, 21 (n=5). The results revealed that the number of MT-ir neurons in the PVN was high during incubation and decreased when hens were deprived of their nests for 6, 18, and 21 days ($P < 0.05$), but the difference was not observed within the SOv and POM between INC and ND groups. This study demonstrated, for the first time, the association between the MTergic system and incubation behavior in the SOv, POM and PVN, suggesting that the induction and maintenance of incubation behavior might be, in part, regulated by the MTergic input from the PVN.

P763**Board Number: B1069****Catecholaminergic Receptors in Peripheral Blood Mononuclear Cells: Opening the Pandora Box of Inflammatory Obesity?.**F. Leite^{1,2,3}, A.H. Santos^{1,2,3}, M. Lima^{2,3}, M. Legnaro⁴, F. Marino⁴, M. Cosentino⁴, L. Ribeiro^{1,5};¹Department of Biochemistry, Univ Porto (FMUP), Porto, Portugal, ²Clinical Haematology, Centro Hospitalar do Porto, Porto, Portugal, ³UMIB/ICBAS - Unit for Multidisciplinary Investigation in Biomedicine, Instituto de Ciências Biomédicas Abel Salazar, Porto, Portugal, ⁴Center of Research in Medical Pharmacology, University of Insubria, Varese, Italy, ⁵Medical Education and Simulation, Faculty of Medicine of the University of Porto,, Porto, Portugal

It is not clear at present which neuroendocrine regulators or metabolic factors affect the immune system in inflammatory obesity. Catecholamines (CA) regulate both metabolism and immune response. CA are synthesized by immune cells and modulate their function through adrenergic receptors (AR) and/or dopaminergic receptors (DRD1-5). The expression of both AR and DRD in immune cells under an obesogenic state is unknown. We hypothesize that the adrenergic and dopaminergic systems present in peripheral blood mononuclear cells (PBMCs) are important neuroendocrine regulators in inflammatory obesity.

We studied 63 blood donors considering waist circumference (WC), lipid profile, plasmatic CA and leptin. Monocyte subpopulations were investigated by flow cytometry using CD14, CD16, CD11b and CD36 markers. β 2-AR (n=60), DRD1-5 and tyrosine hydroxylase (TH) (n=30) expression was evaluated in PBMCs by real-time PCR. Ratio (R) between DRD and TH mRNA expression between individuals with and without central obesity (CO) was calculated. R <0.5 was considered under and >2.0 over expression. CO was defined according to the International Diabetes Federation criteria by using WC (\geq 80 cm for women and \geq 94 cm for men).

CO individuals showed higher plasmatic levels of total cholesterol (p=0.031), VLDL-cholesterol (p=0.023), triacylglycerol (p=0.034) and leptin (p<0.001) compared with non-centrally obese. CD16+ monocytes counting was similar in both groups, but showed lower cellular complexity (p=0.014) and lower CD14 expression (p=0.025) in CO. CO group showed lower β 2 AR expression comparatively to non CO group (p=0.002). While DRD1 was undetected in PBMCs, no differences were found in DRD3 and DRD4 expression between groups. DRD2 (R=0.25; p=0.001), DRD5 (R=0.13; p=0.001) and TH (R=0.43; p=0.004) were under expressed in CO in comparison with non-CO. DRD2 expression was positively correlated with both TH (r=0.771; p<0.001) and CD11b (r=0.927; p<0.001). VLDL, triacylglycerol and leptin plasmatic levels correlated negatively with the expression of DRD2 in PBMCs. In CO, DRD2, DRD5 and TH expression positively correlated with the plasmatic levels of HDL-cholesterol [r=0.605; p=0.013), (r=0.689; p=0.003), (r=0.500; p=0.049), respectively].

Taken together, these results suggest that β 2 AR, DRD2 and DRD5 under expression on PBMCs are associated with a higher inflammatory phenotype of peripheral immune cells and with a more atherogenic lipid profile in inflammatory obesity. Characterizing the involvement of CA in the cross-talk

between adipose tissue and immune cells will likely provide novel clues to the understanding of the mechanisms leading to obesity-associated chronic inflammation.

P764

Board Number: B1070

Hyperhomocysteinemia promotes CD40-CD40L mediated inflammatory monocyte induction in cardiovascular disease via DNA hypomethylation.

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Hyperhomocysteinemia (HHcy), which is indicated by elevated plasma concentration of Hcy (homocysteine), a common risk factor for atherosclerosis, similar to smoking and hyperlipidemia. Previously we reported that Hcy elevates inflammatory monocyte (MC), promotes recruitment of inflammatory MC into atherosclerotic lesion, and contributes to vascular inflammation in animal model of HHcy. The mechanisms mediating the induction of inflammatory MC in human blood are not clear. Blood cell from cardiovascular disease (CVD) patients (n=28) and healthy donor (n=14) were assessed for phenotype characterization by flow cytometry. We found that Hcy, inflammatory MC are increased, positively correlated with CD40+ MC, negatively correlates with Hcy metabolites; S-adenosylmethionine/S-adenosylhomocysteine (SAM/SAH ratio), an indicator of methylation. Hcy produced inflammatory cytokines; IL-6, and TNF α , and synergistically upregulated CD40, reduced CD40 transcription and DNA methyltransferase 1 activity in cultured human MC. We identified two IFN γ /TNF α -responsible DNA hypomethylation CpG dinucleotides at p65 and PU.1 transcription factor consensus sequences on CD40 promoter in white blood cells isolated from CVD patients by Bisulfite pyrosequencing. Triggering CD40 by CD40 ligand (CD40L) induced while blocking CD40L ligation suppressed inflammatory MC development in cultured peripheral blood mononuclear cells. These findings suggest that Hcy potentiates inflammatory cytokine mediated-CD40 induction via CD40 DNA hypomethylation and inflammatory MC induction after binding of CD40 by CD40L.

Defining Therapeutic Targets and New Therapeutics

P765

Board Number: B1071

A phloroglucinol and 3,4-(dihydroxyphenyl) acetic acid fraction with Vitamin C mediates the killing of *Trypanosoma brucei brucei* in vivo and ameliorates the parasite-associated pathological changes.

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The effects of a combined administration of a low dose phloroglucinol and 3,4-(dihydroxyphenyl) acetic acid-rich fraction of *Khaya senegalensis* (pfks) and vitamin C on the severity of *Trypanosoma brucei brucei* infection was investigated. Rats were intraperitoneally infected with *T. brucei brucei* and orally treated with a combination of pfks (100 mg/kg BW) and vitamin C (100 mg/kg BW). At the end of the experiment, the combined treatment demonstrated potent antitrypanosomal effects because it almost eliminated the parasites from the bloodstream (0.87 ± 0.09 (x10⁸) trypanosomes/mL) of the infected animals compared to infected untreated control (38.76 ± 4.21 (x10⁸) trypanosomes/mL). Furthermore, the trypanosome-induced pathological alterations such as anemia, hepatic and renal damages were reversed to near-normal by the combined treatment. Data from the study suggest that the combined treatment of a low dose of pfks and vitamin C is therapeutically potent as antitrypanosomal regimen and could effectively ameliorate the trypanosome-induced anemia and organ damage.

P766

Board Number: B1072

Sox9 positive cells contribute renal epithelial cell regeneration and kidney fibrosis development.

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Sox9 is one of the important transcription factors controlling cell proliferation, fate and differentiation in the development of many tissues and organs. Here we examined the role of Sox9 in renal epithelial cells.

To test the role of Sox9 in renal epithelial cell, we generated mice with tubule epithelial specific Sox9 deletion, by intercrossing the PAX8 rtTA-TetO cre and Sox9 floxed animals. In addition, we also isolated Sox9 positive cells from the mouse kidney to examine the proliferation and differentiation capacities in vitro.

To define the transcription factor related to progenitors in the kidney, we isolated tubule epithelial cells which showed proliferation and differentiation capacity in vitro. We performed genome wide gene expression analysis using microarrays and found cells that show high proliferation and differentiation

capacity had high Sox9, Lgr4, Foxd1 and Pax8 expression. In control mouse and human kidneys, Sox9 is rarely detected in the tubules but in injury models its expression is highly increased most tubules, especially dilated proximal tubules. To test whether Sox9 contributes to kidney regeneration we generated mice with tubule specific deletion of Sox9. These animals developed more severe injury after folic acid injection than wild type animals. Fibronectin, collagen1a and 4a were significantly increased and proliferation markers, cyclin A, B, D and E were remarkably decreased in Sox9 deleted kidney. Similar results were observed in different time courses, 4 weeks and 16 weeks after folic acid injection. In particular, we isolated Sox9 positive cells from mouse kidney and they showed the ability to self-renew and expressed progenitor cell markers. They also showed multi lineage differentiation potential especially renal tubule cells under the specific condition in vitro.

In summary, we demonstrated that Sox9 plays an important role in proliferating and regenerating the renal tubule epithelial cells. Deletion of Sox9 leads decreases proliferation and induce fibrosis development. Sox9 positive cells may represent progenitor cells in the regenerating kidney.

P767

Board Number: B1073

Single-pedicled fasciocutaneous flap survival in ageing rat model of voluntary cardiovascular exercise.

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Impact of cardiovascular voluntary exercise on signaling events underlying composite flap survival was examined in twelve young adult (2 month old) and twelve older adult (6 month old) Sprague-Dawley rats that either had access to running wheel (exercising group, n=6) or not (resting group, n=6) prior to surgery, where a 3:8 width to length ratio pedicled fasciocutaneous flap based on the inferior epigastric artery was raised in each rat and rotated into a defect created in the ventral surface. Histological Hematoxylin and Eosin staining was performed on the flap segments of the biopsies taken on 7th post-operative day (POD). Tissue from the contralateral recipient site was snap frozen and used as baseline (day 0) for Western Blotting data comparison with the biopsies taken on 2nd, 5th and 9th POD. Endogenous VEGF-A, and FGF-2 protein expression, PARP cleavage and activating phosphorylation of Akt, STAT3, p38 MAPK and ERK1/2 were measured in proximal, middle and distal segments of the harvested flaps to reflect the pro-angiogenic, inflammatory, anti-apoptotic, stress, motogenic and mitogenic cellular responses. β -Actin, α -Tubulin, Grb2 and/or GAPDH levels were detected in the same loading of protein-adjusted lysates and used for signal normalization. Young and older exercising rats had significantly higher flap survival rates than their resting counterparts, which coincided with overall elevated VEGF-A levels, stronger PI3-kinase/Akt and MAPK activation and decreased apoptotic PARP cleavage, suggesting that exercise shortens inflammatory phase of wound healing and stimulates flap cell proliferation and motility. Moreover, young resting rats had higher basal VEGF-A levels than resting

old rats. Compared to the resting group, FGF-2 expression and tyrosine phosphorylation of STAT3 was augmented in all flap segments of exercising young adult rats, but there was no significant difference between resting and exercising older adult rats. Given multiple reports on the positive feedback of STAT3 to wound healing response, as well as the potential of FGF-2 to synergize with VEGF-A in promoting neo-angiogenesis and thus better nutrient/oxygen supply, our results may explain why the flaps of younger rats integrate better within the surrounding tissue than those of old rats.

P768

Board Number: B1074

neuroprotective and vasculoprotective microRNA-195 in stroke treatment.

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Acute ischemic stroke has been a major cause of death and the most important factor for disability worldwide. To protect ischemic but still viable neurons from apoptosis may reduce the impact from stroke. Currently, tissue plasminogen activator (tPA) is the only FDA approved drug to treat acute stroke. However, tPA does not have neuroprotective effect and tPA may also cause brain hemorrhage, which restrict its clinical use. We identified a neuroprotective and vascular protective microRNA called microRNA-195 (miR-195).

Two miR-195 binding sites were predicted in the 3'UTR of mRNA of the sema3A gene. Using the reporter assay, we experimentally confirmed these two binding sites. In vivo studies demonstrate that sema3A can influence several down-stream genes including up-regulation of CCND1, Cdc42, FasL, caspase-3, and down-regulation of Bcl-2. Our cellular studies show that sema3A levels are increased and miR-195 levels are decreased when the neuron cell line (SH-SY5Y) is damaged by oxygen-glucose deprivation (OGD). Furthermore, neuron cell viability was reduced by 35% after 3-h OGD and by 55% after 6-h of OGD. If miR-195 was transfected into the cell at 3h and 6h of OGD, a dose-dependent effect on rescuing the OGD-damaged cells was observed.

Previously we have reported that miR-195 has vascular protective effects by reducing inflammation. Now we further identify that miR-195 can suppress an NFkB upstream membrane receptor, which leads to inhibit NFkB pathway. In addition, miR-195 also directly target a component of NFkB complex. Interestingly, an NFkB binding site has been discovered in the promoter of miR-195 gene, and NFkB acts as a negative transcription factor for miR-195. Therefore, NFkB and miR-195 have reciprocal regulation.

To further test the clinical application of miR-195, ischemic stroke is induced in male SD rats by middle cerebral artery occlusion (MCAO) for 24 hours. Formulated miR-195 is injected to the tail vein after the onset of stroke. The animals are killed at 24h and brain infarct sizes were measured. The infarct size can be reduced by 30-40% when miR-195 is injected by 4.5h after stroke. Even though the treatment is at 6h

after stroke, infarct size can be still reduced by 20%. Noticeably, the major limitation of tPA in treating ischemic stroke is the concern of brain hemorrhage, but miR-195 has no such a side effect.

In conclusion, our cellular and animal data suggest miR-195 can be used a therapeutic agent to treat acute ischemic stroke. The lack of hemorrhage side effect and the longer therapeutic window between onset of stroke and miR-195 treatment make this molecule as a promising new drug.

P769

Board Number: B1075

Expression of semaphoring 3F in rat retinal ganglion cells after optic nerve crush.

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Damage to the optic nerve in mammals induces degeneration and apoptosis of the retinal ganglion cell (RGC). Although many factors have been recognized to mediate RGC degeneration in glaucoma, but, the mechanism and process are largely unknown. Semaphorin are a family of glycoproteins that play an important role in repulsive axon guidance during embryogenesis, also, it has been reported to the functional involvement in neuronal apoptosis. We have now investigated that the expression of class 3 semaphorins, and the role of semaphorin in rat RGC after optic nerve crush.

Optic nerve crush injury was induced in Long avans rats. Eyes and retinas were collected at intervals of 3, 7 and 14 days after procedure. Aa a results, we found a decrease in semaphorin 3F levels in the retina at 7 and 14 days after optic nerve crush, but no change in others semaphorin 3 class (sema 3A, 3B or 3C). Particularly, these decreasing expression was seen in the retinal ganglion cell layers of retina. These events were also shown in retinal organ culture using retinas after optic nerve crush and control. These results suggest that neural guidance gene semaphorin 3F may play an important role in the regulation of retinal ganglion cells functions and provide more information about RGC survival.

P770

Board Number: B1076

Ubiquitin-Proteasome Pathway Activity in Cultured Oral Mucosal Epithelial Cell Sheet Grafts Used for Treatment of Experimental Limbal Stem Cell Deficiency in Rabbits.

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Cultured oral mucosal epithelial cell sheets (COMECS) have been proven successful in reconstructing the ocular surface. Harvested from a temperature responsive culture ware, COMECS quickly adhere and reepithelialize the cornea surface, thus speeding up wound healing process. The mechanism of action of

COMECS is complex. Our present study focused on characterizing COMECS ubiquitin-proteasome pathway to determine its role in the therapeutic effects of COMECS. Corneal epithelial cells scraped off the ocular surface of rabbits with limbal stem cell deficiency (LSCD, 3 months post limbectomy) showed a significant decrease in the proteasomal chymotrypsin-like, trypsin-like and caspase-like activities, while COMECS and normal corneal epithelial cells without experimentally-induced LSCD showed significant higher activities. The histological analysis also showed that COMECS stained positive for proteasome subunits beta5, beta1 and Rpt4, an ATPase subunit involved in the ubiquitin proteasome pathway responsible for clearance of damaged and aggregated proteins. Western blot analysis quantified the amount of proteasome subunits and our results showed that COMECS has higher levels of proteasome beta5, as well as higher levels of alpha subunits of the 20S proteasome, when compared to LSCD corneal epithelial cells. The ubiquitin-proteasome pathway was also histologically examined in cornea tissue sections of rabbits grafted with autologous COMECS. Results showed that grafted cornea stained positive for beta5 as well as for Rpt4. Similar to normal healthy corneas, grafted corneas showed that beta5 staining was localized in the cytoplasm and to a lower extent in the nucleus of basal cells. In contrast, corneas with induced LSCD that did not receive COMECS grafts markedly stained in the nucleus, suggesting a nuclear translocation of proteasome in the diseased cornea. In conclusion, our results showed that COMECS include higher levels of proteasome activities compared to LSCD cornea epithelial cells, indicating that COMECS grafting improves or restores the function of the ubiquitin proteasome pathway that is lacking in diseased cornea epithelium with LSCD. Supported by CellSeed Inc. and Emmaus Medical Inc.

P771

Board Number: B1077

AUTOLOGOUS MESENCHYMAL STEM CELLS APPLIED ON DIABETIC FOOT ULCER AS A TREATMENT BY REGENERATIVE MEDICINE.

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Mesenchymal stem cells (MSC) are adherent cells which come from several tissues. These cells have the capability of differentiation to chondrocytes, adipocytes, myoblasts and osteoblasts (plasticity). In vitro expansion in large scale with high viability, make of them suitable candidates in clinical application in the field of regenerative medicine and cell therapy for improve structural and functional tissues. In Mexico diabetes is one of the more important health problem with economic loss due incapacity of worker people and treatment for this has poor successful and finally the patient has loss of limb. In another hand in accordance with the criteria of ISCT (international society for cellular therapy), autologous MSC could be a suitable alternative for tissue regeneration and healing the damage produced in diabetic foot. In this work we showed two cases where autologous MSCs under criteria of ISCT were applied. MSCs

were cultured under quality conditions avoiding the use of SFB. Cells were applied in third passage and it was carry of wound healing during seven months. Evident regeneration and a partial functionality recover were showed in the patients treated with MSCs indicating that this procedure could be implemented as a treatment in lesions with severe damage and not only in diabetic foot. Mechanisms involved in the regeneration by the MSCs are now studied by our group of work.

P772

Board Number: B1078

Effect of dehydroepiandrosterone and (-)-epicatechin in the expression of BMP2, SPARC and RUNX2 in Mesenchymal Stem Cell from human Bone Marrow.

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Introduction: Osteoporosis is a disease which is characterized by loss of bone and susceptibility to fractures¹. Although it has been applied different treatments most of them have important side effects and little benefits². On the other hand, has been demonstrated that antioxidants like dehydroepiandrosterone (DHEA) and (-)-epicatechin (EC) of green tea can improve bone density of menopausal women³. For this reason, in this work we studied the effect of DHEA and EC over expression of osteogenic genes (SPARC, RUNX2 and BMP2) in human Bone Marrow Mesenchymal Stem Cells (hBM-MSC). Methodology: hBM-MSCs were isolated from healthy people and characterized according the International Society for Cell Therapy standards, after that the cells were treated with different combinations of DHEA and EC for seven days and total RNA were obtained and used for final point PCR. Results: Ours endogenous gene (GAPDH) were expressed in all samples, in the same way SPARC and RUNX2 were present in all conditions. BMP2 was absent in the negative control (DMEM-F12 medium) and present in the positive control (osteogenic medium), while the cells with DHEA 1, 10 and 100uM express BMP2. When DHEA was mixed with 100uM of EC the expression was less, but DHEA 1, 10 and 100uM with 1uM of EC, BMP2 was inhibited. But when we mix DHEA 10 and 100 with 10uM of EC the expression of BMP2 were increased and it was absolutely inhibited with DHEA 1uM and EC 10uM. EC at 1 and 100uM induced BMP2 expression but not at 10uM. Conclusions: EC and DHEA in low concentrations induce expression of osteogenic gene (BMP2), which could result in improvement of bone density; this could be useful in the treatment of different bone diseases not only in osteoporosis. References: 1.- Crandall et al., Treatment to Prevent Fractures in Men and Women with Low Bone Density or Osteoporosis: Update of a 2007. Report. 2012. 2.- Zhang et al., Osteoporos Int 2014 Jan; 25 (1): 317-24; Guggina et al., Bone. 2012 Dec; 51(6): 975-80; NBGH Update of a 2007 Report. May 2012. 3.- Wang et al., Chin Med J (Engl). 2012 Apr; 125(7): 1230-5; Devine et al., Am J Clin Nutr 2007; 86:1243-7; Hegarty et al., Am J Clin Nutr 2000; 71: 1003-7.

P773**Board Number: B1079****Fluorescence lifetime FRET measures transferrin tumor uptake using in vivo small animal imaging: Implications in the development of targeted drug therapy for estrogen receptor positive breast cancer.**A. Rudkouskaya¹, S. Rajoria¹, L. Zhao², X. Intes², M.M. Barroso¹;¹Cardiovascular Sciences, Albany Med Coll, Albany, NY, ²Biomedical Engineering, Rensselaer Polytechnic Institute, Troy, NY

Traditional cancer therapy generally leads to harmful side effects, thus warranting development of targeted therapies, which are better tolerated by cancer patients. Our goal is to develop *in vivo* non-invasive optical imaging assays for optimization of anti-cancer drug targeted therapy. We have established a fluorescence lifetime Förster Resonance Energy Transfer (FL-FRET) non-invasive whole-body *in vivo* imaging technique that can discriminate bound and internalized near-infrared (NIR)-labeled transferrin (Tfn) from free, soluble NIR-Tfn. The FRET-based assay exploits the homodimeric nature of transferrin receptor (TFR) that binds two molecules of Tfn in close proximity to determine dimerization and internalization of TFR-Tfn complexes into cancer cells. The Tfn FRET assay has been validated *in vitro* by visible intensity-based confocal microscopy and visible and NIR fluorescence lifetime microscopy (FLIM). Our approach provides a new method to optimize targeted drug therapy by accurately quantitating *in vivo* the tumor uptake of NIR-Tfn-drug conjugates, since the expression of TFR is upregulated in a wide variety of human tumors. T47D human breast cancer cells, a model of estrogen receptor positive (ER+) human breast cancer, show the highest TFR expression and Tfn uptake in comparison to those of normal human mammary epithelial cells (HMEC), suggesting that Tfn can be used as specific targeting ligand to ER+ breast cancer cells. FL-FRET tomographic imaging *in vivo* has been used to measure the internalization of tail-vein injected NIR-Tfn into human breast T47D tumor xenografts in live nude mice. Immunohistochemistry of tumor sections suggests that the injected NIR-Tfn is translocated to the tumor site. Quantification of FRET donor % (FD%) in T47D tumor xenografts *in vivo*, indicates a higher proportion of FD% with increasing acceptor:donor ratio, demonstrating tumor uptake of NIR-Tfn. Tfn uptake concentration curve shows high sensitivity of FL-FRET imaging using NIR-Tfn as low as 10µg/ml of blood. Furthermore, relative high FD% for holo-Tfn (iron-loaded) compared to that of apo-Tfn (iron-depleted) demonstrates specific TFR-mediated uptake of holo-Tfn by T47D tumor xenografts, as expected since iron-depleted apo-Tfn shows reduced binding affinity towards TFR. Recently, NIR-Tfn-oxalate, an iron release chemical mutant, has been tested in our tumor imaging assay. Elevated FD% levels of NIR-Tfn-oxalate are detected in T47D tumor xenografts in comparison to those of holo-Tfn, indicating increased NIR-Tfn-oxalate tumor cellular accumulation. In conclusion, our novel NIR FL-FRET assay successfully demonstrates the quantitative receptor-mediated uptake of Tfn into human breast tumors *in vivo* using a novel non-invasive NIR FL-FRET imaging assay.

P774**Board Number: B1080****Substance P modulates essential properties of murine bone marrow-derived stromal cells.**M.J. Dubon¹, N. Jung², J. Um¹, Y. Son^{3,4}, K. Park⁵;

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Bone marrow-derived stromal cells (MSCs) have self-renewal characteristics, multilineage differentiation potential and are able to mobilize from the bone marrow (BM) into peripheral tissues where they promote wound healing and tissue repair. Previous studies demonstrate that substance P (SP) induces the proliferation of MSCs *in vitro* and promotes the migration of MSCs from the BM to injury sites. The aims of this study were to evaluate 1) the effects of SP on the homeostasis of MSCs within the BM, 2) whether SP can affect the differentiation potential of MSCs, 3) the effects of SP on factors that could be involved in the regulation of cell trafficking between the BM and the periphery, and 4) the mechanism involved in the mobilization of MSCs. Furthermore, we investigated the effects of SP on the proliferation and migration potential of MSCs *in vitro* using two mouse-derived MSCs like cell lines (OP9 and ST2) and searched for the mechanisms involved in the SP-mediated proliferation and mobilization of these cells. We found an increase in the CFU-F forming population of total bone marrow cells 2 days after SP injection. The mRNA levels of osteogenic markers (osteopontin and type 1 collagen) increased 1 day after SP injection, suggesting that SP induces the differentiation of MSCs towards this lineage. We also found an increase in the mRNA levels both of N-cadherin and SDF-1 at 1 day after SP injection which suggests their involvement in the mobilization of MSCs mediated by SP and in the regulation of stem cell trafficking between the BM and the periphery, respectively. Substance P induced the migration potential of ST2 cells and increased the proliferation of OP9 cells. Overall, these data suggests that SP has the ability to modulate certain properties of MSCs linked to their therapeutic potential and trafficking. Yet, the mechanisms involved in the SP-mediated proliferation and mobilization of MSCs remain to be explored in further experiments. (Supported by NRF-2012M3A9C6050499 and NRF-2012R1A1A2042265)

P775**Board Number: B1081****The effect of Mild Electrical Stimulation with Heat Shock on kidney dysfunction in type 2 diabetic KKAY mice.**Y. Kai¹, R. Fukuda¹, K. Omachi¹, Y. Okamoto¹, M. Suico¹, T. Shuto¹, H. Kai¹;¹Molecular Medicine, Kumamoto Univ Sch Pharm, Kumamoto, Japan

Diabetic Nephropathy (DN) is the most common cause of end-stage renal disease, but the treatment to decelerate the progression of DN is still inadequate. We previously showed that mild electrical stimulation (MES) with heat shock (HS) treatment ameliorated insulin resistance and suppressed some inflammatory cytokines in diabetes mouse models by enhancing insulin-stimulated Akt-phosphorylation. Furthermore, we showed MES+HS restrained the progression of albuminuria/proteinuria and nephritis in Col4a5 mutant mouse, which is a mouse model for hereditary glomerulopathy. However, it is uncertain whether MES+HS ameliorates kidney dysfunction in diabetes. In this study, we evaluated the effect of MES+HS on kidney dysfunction in type 2 diabetes model KKAY mice. Although, MES+HS did not affect the progression of albuminuria/proteinuria and renal injury in KKAY mice, MES+HS tended to suppress pro-inflammatory cytokines (IL-6, IL-1 β), and kidney injury marker (Lcn-2) in kidney. Furthermore, the kidney weight tended to be suppressed in MES+HS-treated mice. These results suggest that MES+HS improves renal hypertrophy in DN. Additionally, MES+HS improved insulin sensitivity assessed by intraperitoneal insulin tolerance test. In MPC-5, MES+HS suppressed high glucose-induced apoptosis via p38 pathway. Collectively, MES+HS improved not only diabetic phenotype but also kidney dysfunction in KKAY mice. Our data suggested that MES+HS has beneficial effect on kidney and MES+HS could be a novel therapeutic strategy for the amelioration of DN pathologies.

P776**Board Number: B1082****Substance P delays onset of type 1 diabetes.**J. Um¹, N. Jung², S. Choi³, Y. Son^{1,3}, K. Park⁴;¹Graduate School of Biotechnology, Kyung Hee University, Yongin, Korea, ²Graduate school of Biotechnology, Kyung Hee University, Yongin, Korea, ³Department of Genetic Engineering, Kyung Hee University, Yongin, Korea, ⁴East-West Medical Research Institute, Kyung Hee University, Seoul, Korea

Pancreatic islet in type 1 diabetes is characterized with the decrease in β -cell mass following autoimmune destruction. In this study, we investigated whether substance P (SP) can delay the onset of diabetes in non-obese diabetic (NOD) mice as SP plays key roles to modulate immune response. When SP (5 nmoles/kg) had been intravenously administered into NOD mice for 14 weeks or 21 weeks, hyperglycemia was delayed. Consistent with this result, SP preserved β -cells at the time points. Moreover, we found that SP increased β -cell proliferation in vitro. SP group also decreased significantly in infiltration degree of immune cells such as CD3 positive cells in comparison to the control mice. Furthermore, as we assumed that SP could systemically affect immune modulation, we analyzed

immune infiltration into salivary glands of NOD mice. SP treatment reduced immune infiltration into salivary glands. Collectively, it is likely that SP might play inhibitory roles in the progress of type 1 diabetes through the maintenance of β -cells homeostasis and the systemic immune modulation. (Supported by NRF-2012M3A9C6050499, NRF-2012R1A1A2042265, and HI13C1479)

P777

Board Number: B1083

Substance P preserves pancreatic endocrine β -cells in type 2 diabetes.

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A decrease in pancreatic β -cell population may contribute to the progress of type 2 diabetes. It has been reported that substance P (SP) can regenerate damaged tissues. Therefore, we investigated the effects of SP on the regeneration of β -cell population in db/db type 2 diabetic mice. For this purpose, mice were injected with SP (5 or 50 nmoles/kg) twice a week for 12 weeks. SP treatment reversed hyperglycemia in db/db mice. Interestingly, SP preserved population of β -cells in pancreas of db/db mice. It is also well known that pancreatic satellite cells (PSCs) are activated in response to pancreatic injury and the activated PSCs express α -smooth muscle actin (α -SMA). Significantly, the activated PSCs decrease mass of β -cells. We found SP reduced the frequency of intra-islet α -SMA positive cells in db/db mice. In our previous works, SP increases the cellular proliferation of β -cell in response to pancreas injury. Based on these results, we hypothesized whether SP can increase the cellular proliferation of β -cells for maintenance of β -cell homeostasis in db/db mice. To examine this hypothesis, we carried out BrdU incorporation assay by use of MIN6 cell line under high glucose condition. Importantly, SP increased the cellular proliferation of MIN6 cells. Taken together, it is very likely that SP might maintain β -cell homeostasis in type 2 diabetes through the increased proliferation of β -cells. (Supported by NRF-2012M3A9C6050499, NRF-2012R1A1A2042265, and HI13C1479)

P778

Board Number: B1084

IDOL Inhibitors for the Treatment of Hypercholesterolemia.

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Despite the widespread use of statins and other approved therapeutics, hypercholesterolemia represents a significant medical issue for a large group of patients unable to effectively regulate their cholesterol levels, contributing to significant morbidity and mortality. Cholesterol uptake is primarily mediated by the hepatic low density lipoprotein receptor (LDLR) which binds and internalizes plasma

LDL. Elevated levels of LDLR are associated with reduced LDL levels and vice versa. Recently, the E3 ligase IDOL (Inducible Degradator of LDLR) was shown to be a key regulator of LDLR protein levels. IDOL is a unique E3 ligase that utilizes its FERM domain to specifically target LDLR for polyubiquitylation and subsequent lysosomal degradation. Genetic ablation of IDOL results in elevated levels of LDLR protein. Thus, inhibition of IDOL may be a beneficial approach for the treatment of hypercholesterolemia.

The ubiquitin-proteasome system provides a rich landscape for drug discovery. E3 ligases in particular are considered attractive therapeutic targets in a wide variety of disorders including metabolic disorders. The recent discovery that the IMiD class of drugs targets Cereblon E3 ligase provides the therapeutic validation of the E3 ligases. However, discovery and development of E3 ligase inhibitors as first in class clinical candidates remain challenging.

Here we report that using our UbiPro™ drug discovery platform we have discovered novel IDOL inhibitors that modulate cellular cholesterol homeostasis. Specifically these compounds increased LDLR levels and increased LDL association in various cellular models. Biophysical characterization revealed direct binding of the compounds to IDOL and perturbation of IDOL:LDLR interactions. The most promising compounds were used as starting point to develop novel drug like molecules and the efficacy of the lead compounds is being evaluated in translational models of hypercholesterolemia. Data will be presented summarizing our progress to date targeting IDOL for the treatment of hypercholesterolemia.

P779

Board Number: B1085

Apomine, an inhibitor of cholesterol synthesis, increases secretion of apolipoprotein E from human astrocytoma and liver hepatocyte cells.

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Apolipoprotein E (apoE) genotype is the biggest known risk factor for Alzheimer's disease (AD). ApoE is an important protein involved with cholesterol homeostasis and other AD-associated processes. Apomine is a potent cholesterol-reducing compound that possibly mediates its actions through proteolytic degradation of HMGCoA-reductase (the rate-limiting enzyme in cholesterol synthesis) and may affect apoE levels. We hypothesize that cells treated with Apomine are programmed to increase apoE secretion to capture and internalize more cholesterol through lipid membrane receptors from external sources to compensate for the loss of HMGCoA-reductase activity.

Treatment of human CCF-STTG1 astrocytoma with Apomine in serum-free medium leads to a significant increase in secreted apoE (1.5-fold; determined by ELISA). Despite the increase in apoE secretion, there was not a significant increase in apoE mRNA expression (as determined by qPCR) in CCF-STTG1 cells. Consistent with our hypothesis, we found that Apomine increases expression of mRNA for several surface apoE receptors. This may be in-part due to Apomine-mediated decrease in inducible-degrader of

low-density lipoprotein receptor (LDLR) mRNA levels. This suggests that CCF-STTG1 cells will have increased levels of cell surface lipid receptor proteins, in preparation to uptake cholesterol-bound apoE.

Apomine-treated HepG2 liver hepatocyte cells also yielded increased levels of secreted apoE in serum-free medium (1.75-fold). Apomine treatment also increased mRNA levels for low-density lipoprotein receptor (LDLR) and very-low density lipoprotein receptor (VLDLR), but not other apoE receptors. However, in contrast to CCF-STTG1 cells, apoE mRNA was increased two-fold. This suggests that HepG2 cells will have increased apoE protein production and cell surface lipid receptor proteins to facilitate import of apoE-bound cholesterol.

This increase in secreted apoE was completely blocked if the HepG2 cells were treated with Apomine in bovine growth serum-containing medium. These results indicate that the presence of cholesterol in the growth medium suppresses the cell's drive to search for cholesterol through increased apoE secretion. These data suggest that Apomine regulates apoE expression at both the transcriptional and post-translational levels. This novel regulation of apoE expression by Apomine is relevant to the fields of AD and other cholesterol homeostasis related processes.

P780

Board Number: B1086

Defining genetic pathways of disease through genetic suppression screens in *Caenorhabditis elegans*.

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There are approximately 7,000 rare diseases known in humans, many of which are monogenic (caused by a mutation in a single gene). *C. elegans* has orthologs of approximately 50 percent of the genes responsible for human diseases. We are using mutants in these orthologous genes in *C. elegans* to perform EMS mutagenesis suppressor screens to find genes that are involved in various disease pathways. This will provide insight into the genetic and molecular mechanisms of these diseases, offering possible therapeutic targets. We are using mutants in the *C. elegans* type IV collagens and the ubiquitin-activating enzyme in suppressor screens to identify potential novel therapeutic targets.

The ubiquitin-activating enzyme in human (UBA1 or UBE1) and in *C. elegans* (*uba-1*) is required for activation of ubiquitin for downstream ligation of ubiquitin by the ubiquitin ligases and subsequent substrate degradation by the proteasome. Mutations in human UBA1 cause early-onset spinal muscular atrophy and are involved in some cancers. UBA-1 is the only known ubiquitin-activating enzyme in *C. elegans* and the temperature sensitive (*ts*) *uba-1(it129)* allele causes embryonic lethality, larval lethality and sperm-specific sterility at restrictive temperatures.

We have obtained suppressors of the larval arrest and embryonic lethality phenotypes in *ts* alleles at restrictive temperatures of *emb-9*, *let-2* and *uba-1*, and we are currently in the process of doing whole genome sequencing to identify the genes responsible for suppression. One promising candidate for

suppression of the emb-9(hc70ts) allele is dig-1, a giant member of the immunoglobulin superfamily. We are currently testing this and other candidates to confirm their identities as suppressors.

We plan to continue to characterize the suppressor mutations as well as the genetic and molecular nature of the suppressor activity that allows the animals with the mutation to survive. We are also continuing mutagenesis screens to find suppressors of additional disease genes. These results will help further define genetic pathways and potentially identify genes that could serve as therapeutic targets to suppress disease symptoms.

P781

Board Number: B1087

Expanding the therapeutic possibilities of NOTCH3 Cysteine Quantity Correction in CADASIL.

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Cerebral autosomal dominant arteriopathy with subcortical infarctions and leukoencephalopathy (CADASIL) is the most common monogenetic cause of vascular dementia and stroke. CADASIL is caused by highly stereotyped missense mutations in *NOTCH3*, which invariably alter the number of cysteine residues in one of the 34 epidermal growth factor-like repeat (EGFr) domains of the NOTCH3 protein. This results in an EGFr domain with an uneven number of cysteines, which disrupts normal disulphide bridge formation and leads to toxic NOTCH3 accumulation in the vasculature. We hypothesize that re-establishing the correct number of 6 cysteines in an EGFr domain (Cysteine Quantity Correction) leads to restored disulphide bridge formation, thereby counteracting NOTCH3 accumulation. To achieve this Cysteine Quantity Correction, exon skipping is used. In this technique, antisense oligonucleotides (AONs) are used to skip targeted *NOTCH3* exons from pre-mRNA. Previous work has focused on jointly skipping *NOTCH3* exons 4 and 5, which harbour the majority of CADASIL causing mutations. However, at least 12 *NOTCH3* exons are eligible for this exon skipping approach. The current study aims to expand the exon skip approach to other eligible *NOTCH3* exons. AONs targeting these additional exons were transfected into fibroblasts and CADASIL patient derived vascular smooth muscle cells. Isolated and joint exon skipping were successfully observed in *in vitro* experiments. Transient transfection of cDNA deletion constructs lacking the targeted exons showed that modified NOTCH3 proteins are formed. The exon skip approach to re-establish six cysteines now includes strategies to target five of the twelve eligible *NOTCH3* exons, which collectively harbour 57% of the distinct CADASIL-causing mutations. This work is supported by grants from the Brain Foundation of the Netherlands, grant no. F2009(1)-25, The Leiden University Research Institute (LURIS) exemplification grant, and by ZonMw, The Netherlands Organization for Health Research and Development, grant no. 40-41900-98-018.

P782**Board Number: B1088****Effect of 3,4',5-Trismethoxybenzophenone on Hepatitis C-Virus Expressing Hepatoma cells.**C.J. Patton¹, H.R. Kotturi²;¹Department of Biology, University of Central Oklahoma, Edmond, OK, ²Department of Biology, University of Central Oklahoma, Edmond, OK

Hepatitis C virus (HCV) is a positive strand RNA virus. HCV infection is a leading cause of chronic hepatitis (>80%). Chronic HCV infection is considered to be a prominent risk factor for the development of hepatocellular carcinoma (HCC). Currently, FDA-approved triple therapy for treating HCV infections consists of a protease inhibitor, ribavirin, and pegylated interferon (IFN) which is only partially effective and the success of treatment depends on the host's genetic make-up, ethnicity, viral variants, quasispecies, drug-resistance of HCV strains and tolerability. Hence, there is a growing need for developing safer and inexpensive drugs for treating HCV infections and HCC. Human hepatoma cell line (FCA4 cells) that harbors a subgenomic selectable HCV replicon was used to study the effect of 3,4',5-Trismethoxybenzophenone (TMBP). The Huh7 cell line lacking the replicon served as the corresponding control. Western blot was conducted to analyze the viral NS5B polymerase level in FCA4 cells treated with varying concentrations of the drug. The effect of TMBP on cell proliferation/viability was assayed using MTT method. TMBP was found to be highly effective in reducing NS5B polymerase level at 2.5 μ M, which was found to be below its IC₅₀ as determined by the MTT assay. Dimethylsulfoxide (DMSO) used as solvent showed no inhibitory effect on the NS5B abundance. TMBP also exhibited disruptive effects on the normal architecture of the HCV replicon-expressing cells. Only TMBP, but not DMSO inhibited the growth of 3D spheroids in cell culture. Our results indicate that TMBP is effective in inhibiting HCV replication in liver cells at lower concentration. It also possess anti-tumor properties against liver cancer and has a potential to be used as a therapeutic agent.

P783**Board Number: B1089****Screening of small compounds to develop endoplasmic reticulum-oriented drug for familial amyloid polyneuropathy.**K. Chosa¹, S. Takaki¹, R. Yamakawa¹, Y. Wakita¹, Y. Teranishi¹, T. Sato^{1,2}, T. Yokoyama³, M. Mizuguchi³, M. Suico¹, T. Shuto¹, H. Kai¹;¹Molecular Medicine, Kumamoto Univ Sch Pharm, Kumamoto, Japan, ²Cell Fate Control, Kumamoto Univ Institute of Molecular Embryology and Genetics, Kumamoto, Japan, ³Pharmaceutical Sciences, Toyama Univ, Toyama, Japan

Familial amyloid polyneuropathy (FAP) is one of the hereditary amyloidoses caused by point mutation in the human plasma protein, transthyretin (TTR). Amyloid fibrils derived from TTR variants accumulate in peripheral nerves and visceral organs. TTR variants are easily dissociated from tetramer to monomer because of having low energetic stability of their tetrameric structure in comparison with wild-type (WT) TTR. The only treatment currently available for FAP is liver transplantation. Therapeutic agents that are being developed aim at stabilizing extracellular TTR tetramer. Previously, we demonstrated that endoplasmic reticulum (ER) quality control system and ER-associated degradation (ERAD) of TTR are pathological determinants of FAP. The monomeric mutation introduced in the vast majority of TTR mutants that are normally secreted resulted in the ER retention and efficient degradation of these monomeric mutants by ERAD. Therefore, inhibition of TTR tetramerization in ER lumen and suppression of TTR variants secretion could be a promising therapeutic strategy for FAP. Here we employed two approaches to find tetramerization inhibitor, namely structure-based virtual screening (SVS) and high-throughput screening (HTS). In SVS, we identified some candidate compounds with a potential as destabilizer of intracellular TTR tetramer. The selected compounds were assayed using HEK293 cells stably expressing the most common variant, V30M TTR. Our data indicated that some of these compounds inhibited V30M TTR tetramerization and extracellular secretion of V30M TTR, suggesting a possible use for preventing TTR amyloid formation. In HTS, we checked the compounds that have the ability to dissociate or destabilize TTR tetramer by using ANS assay, which is a cell-free, in vitro method. We utilized the chemical library - the Core Library 9600 preserved at Open Innovation Center for Drug Discovery (OCDD) in Tokyo. The number of compounds showing >30% inhibition was 168 compounds ($S/B = 47.1 \pm 2.2$, $Z' \text{ factor} = 0.92 \pm 0.01$). We also screened the validated compound library in the LOPAC1280 for tetramerization-inhibitory activity. There were 20 compounds with >30% inhibition of TTR tetramerization ($S/B = 14.5 \pm 1.3$, $Z' \text{ factor} = 0.89 \pm 0.01$). The selected compounds will be further evaluated for their effects.

P784**Board Number: B1090****Analysis of miRNA target genes; a new approach to understand the cause of obesity.**M.J. Khan¹, W. Khan¹, A.A. Raja¹, A. Naeem¹;¹Department of Biosciences, COMSATS Institute of Information Technology Islamabad, Islamabad, Pakistan

Obesity is a health risk that leads to numerous metabolic complications. It has been known to cause serious diseases such as Type 2 Diabetes, atherosclerosis, degenerative disorders including dementia, and some types of cancer. Obesity related metabolic syndromes are now prevalent in children as well. Several genetic factors have been reported to cause obesity that is regulated at molecular level. Among the other regulatory factors, MicroRNAs (miRNAs) are partially or completely complementary to the messenger (mRNA) and their main function is to down regulate the gene expression. We carried out a study to explore the effects of miRNAs on differentially expressed genes (DEGs), taken from an experiment on child obesity. miRNA target genes were obtained using FINDTAR3 and the functional analysis was done using Database for Annotation, Visualization, and Integrated Discovery (DAVID). Role of all the miRNAs were observed in biological processes, cellular compartments, molecular functions, protein interaction and binding. Our preliminary results have identified the role of miR-143 in all biological processes including energy metabolism, homeostasis, and cell growth. The recently discovered miR-519d controlled the regulation of metal ion transport and positive regulation of RNA polymerase II promoter. In the category of cellular compartment of DAVID, miR-138 was the major regulator of gene expression in mitochondria. With this analysis, we identified genes and biological processes regulated by particular miRNAs that play crucial role in metabolic dysfunctions leading to obesity; this opens a new dimension for future studies to a better understanding of the cause of obesity.

Key words: obesity, miRNA, gene expression**P785****Board Number: B1091****Implication of TRPM4 channel in ischemia-reperfusion injury.**K. Takahashi¹, K. Naruse¹;¹Cardiovascular Physiology, Okayama Univ. Grad. Sch. Med. Dent. Pharmaceut. Sci., Okayama, Japan

Ischemic heart disease often causes heart failure. During ischemia-reperfusion (I/R), excessive reactive oxygen species (ROS), including hydrogen peroxide (H₂O₂), are produced in cardiac tissue, where they induce cell death. We previously reported that a TRPM4 channel inhibitor 9-Phenanthrol protects cardiac I/R injury in the excised rat heart. Based on this finding, we hypothesized that TRPM4 channels are involved in the pathophysiology of the cardiac I/R injury. We confirmed that intravenous application of 9-Phenanthrol mitigated the development of myocardial infarction caused by the occlusion of the left

anterior descending artery in rats. Positive expression of TRPM4 channels in the ventricular cardiomyocytes was confirmed by immunohistochemistry. To evaluate the toxic effect of ROS on cardiac cells, we measured cellular viability of H9c2 cardiomyocytes underwent H₂O₂ challenge. Pretreatment of 9-Phenanthrol preserved cellular viability. Furthermore, knockdown of TRPM4 channels preserved the viability of H9c2 cardiomyocytes exposed to H₂O₂. These results suggest that TRPM4 channels are involved in development of cardiac ischemia-reperfusion injury.

P786

Board Number: B1092

A novel small molecule antagonist of Id1 inhibits breast cancer metastasis to the lungs.

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Breast cancer metastasis is one of the greatest therapeutic challenges to prolonging patient survival. Thus the identification of genes involved in metastasis is important for a greater understanding of the metastatic process, as well as for identifying clinically relevant targets for which novel therapeutics could be developed. The inhibitor of DNA-binding (Id) proteins have been implicated in breast cancer metastasis, with high Id1 expression being observed at metastatic sites. Furthermore, Id1 has been shown to play a role in mesenchymal-to-epithelial transition (MET), which allows for circulating cancer cells to seed distant metastatic sites, such as the lung. It was previously shown that knockdown of Id1 prevented MET of tumors cells in the lung, and the development of lung metastases in mouse models of breast cancer. Thus targeting Id proteins may be an effective and novel approach for treating and/or preventing metastases in breast cancer patients. Here we present data investigating the biology of a recently developed small molecule antagonist of Id proteins. An *in silico* small molecule screen of 2 x 10⁶ compounds was carried out to identify compounds that would bind a hydrophobic pocket adjacent to the dimerization domain of Id1, which would disrupt dimerization of Id1 with its binding partners, such as E47, and hence its downstream signaling. Of the 15 positive hits, one compound, named AGX-51 (supplied by Angiogenex Inc.) was able to enhance E47 binding to DNA, in a dose-dependent manner, suggesting that AGX-51 antagonizes Id1-E47 dimerization. Treating the murine breast cancer cell line 4T1 with AGX-51 resulted in decreased Id1 protein expression, suggestive of protein degradation. In cell culture assays, AGX-51 resulted in decreased cell viability, as measured by alamar blue assays, and inhibited the formation of mammospheres, consistent with previous Id1 knockdown experiments. To assess the effect of AGX-51 on metastasis, AGX-51 was administered at 50mg/kg via intraperitoneal injection, 24-hours after tail vein injection of 4T1 cells. Development of metastases was monitored using bioluminescent imaging. At two weeks we observed that AGX-51 dramatically inhibited the development of lung metastases. Furthermore, significantly greater inhibition of lung metastases was observed when AGX-51 administration was increased from once to twice daily. We are currently investigating the specific mechanisms of AGX-51's anti-metastatic activity, by investigating the effects of AGX-51 on the 4T1 transcriptome and the EMT/MET pathway. In summary, AGX-51 showed potent anti-

metastatic activity, and is a promising novel therapeutic for metastatic breast cancer, warranting further investigation for translation into the clinic.

POSTER PRESENTATIONS-Monday, December 8

Science Education

P787

Board Number: B100

An interdisciplinary project-based approach using *Drosophila* genetics and cell biology to improve reasoning, problem solving and foundational knowledge in high school students.

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Mastery of foundational scientific concepts, problem solving, and reasoning skills are three areas that have been recognized as needing improvement in both undergraduate and high school students. Here we describe an educational initiative that uses an interdisciplinary approach between a science educator and research scientist to improve student performance in these three areas using project-based instruction. We have used *Drosophila melanogaster* to teach basic biological concepts related to genetics and cell biology to five classes of high school students in the Olathe school district of Kansas. The project was structured around the integration of the new Next Generation Science Standards and includes 7 core concepts: basic biochemistry, cell structure, cell division, molecular genetics, Mendelian genetics, biotechnology, and informatics. In addition to these core concepts, the project aims to improve student performance in the eight practices recognized as being essential for science and engineering students in K-12 in the Next Generation Science Standards including: asking questions, using models, planning and carrying out investigations, analyzing and interpreting data, using mathematics and computational thinking, constructing explanations, using evidence to argue, and obtaining, evaluating, and communicating information. To examine the success of our approach in increasing the students mastery of foundational scientific concepts, we used a pre-test and post-test from the AAAS Science Assessment Project that evaluates the students general knowledge of concepts covered within the scope of our project and many that also lie outside. Our results indicate that the students in our project-based classes scored higher than the national average on questions related both to and outside of the project.

P788**Board Number: B101****Biological Research Through Independent Investigations in a High School Setting.**A. Hoover¹, N. Kumar¹, J. Gleklen¹, M. Fields¹;¹Sidwell Friends School, Washington, DC

Students at Sidwell Friends School in Washington, D.C. are given the opportunity to conduct original research both inside and outside of the classroom. In Biology 1A, an accelerated biology course, ninth grade students conduct an Independent Research Project of their choosing. Later in their high school careers, students who wish to refine their research skills and to learn more advanced concepts in neuroscience may opt to take Sidwell's Neuroscience Research course. Additionally, students wanting to conduct research outside of class may join the Biological Research and Investigations in Neuroscience (BRAIN) Club. Experienced members of the BRAIN club act as mentors for the more junior members while also conducting their own research. The BRAIN Club also created a program referred to as Sci-Fins in which Sidwell students use an inquiry-based approach to tutor students from DC's Cesar Chavez Public Charter School in basic biology. Sidwell students are also provided the opportunity to further branch out in the scientific community by partnering with institutions such as the National Institutes of Health, the Georgetown University Medical Center, and the MAYO clinic. Most students decide to use zebrafish, *Danio rerio*, as a model organism for their projects in order to take advantage of its translucent embryos, rapid development, easy care, and completely mapped genome. Ongoing experiments involving *Danio rerio* include observing the effect of nicotine on the mesolimbic pathway, distinguishing shoaling preferences, evaluating the effect of pregnenolone on cell movement, and determining how ethanol affects a cell-signaling pathway known as the sonic hedgehog pathway, at different stages in zebrafish development.

P789**Board Number: B102****Identification of molecular targets of nutritional supplements in *Drosophila* oogenesis by Immersion Science High School Program Students.**D.M. Ruiz-Whalen¹, A. Bolotsky¹, L. Leonard², C. McAuliffe³, J. Raj⁴, Y. Miglo⁵, N. Archer⁶, C. Pritchard⁷, P. Zaveri², T. Singh¹, E. Boamah¹, T. Strohlic¹, M. Radu¹, R. Henry⁸, A.M. O'Reilly¹;¹Cancer Biology, Fox Chase Cancer Center, Philadelphia, PA, ²North Penn High School, Lansdale, PA,³Council Rock High School, Newtown, PA, ⁴Merion Mercy Academy, Merion Station, PA, ⁵Neshaminy High School, Langhorne, PA, ⁶Garnet Valley High School, Glen Mills, PA, ⁷Souderton Area High School, Souderton, PA, ⁸Cancer Epigenetics, Fox Chase Cancer Center, Philadelphia, PA

Reports on the positive and negative effects of nutritional supplements on health abound in the public media, but little scientific evidence exists to support most claims. Recently, we identified a single

nutrient, dietary cholesterol, that regulates proliferation rates of epithelial Follicle Stem Cells in the *Drosophila* ovary by controlling the levels of the growth factor Hedgehog available to the stem cell niche. This work defines a novel signal transduction pathway that controls FSC function in response to global changes in the external environment of the organism and suggests that a single nutrient can have dramatic effects on an individual cell population. To address the effects of individual nutrients on Follicle Stem Cells and other cellular events during oogenesis, 32 high school students in our newly developed Immersion Science laboratory research training program screened 48 nutritional supplements for effects on egg production in *Drosophila*. Compounds that directly targeted the EGFR resulted in phenotypes indistinguishable from those observed in EGFR mutant flies, demonstrating the efficacy of the assay. Nine additional nutritional supplements scored in the screen, including three that impaired signaling through Extracellular-Signal-Regulated-Kinase (ERK) and Hedgehog signaling pathways in the ovary. Here, we report the effects of two of these supplements on the development of proliferative masses of cells that exhibit extremely elevated levels of ERK signaling and resemble epithelial tumors.

P790

Board Number: B103

Cultivating ensembles across biology education and research.

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A diverse group of cell biology educators and researchers in collaboration with professionals in theater, leadership and human development are cultivating a STEM culture that is highly inclusive, collaborative and innovative. A growing population of Science Technology Engineering and Mathematics students and professionals are increasingly involved in this movement. These professionals have created non-major classes that integrate biology and theater, professional groups that use improvisation to advance their communication skills, and performance based workshops to support professional skill development for women and people from traditionally underrepresented groups. A common root in these efforts is their activity to cultivate ensembles- groups whose work is qualitatively different than the sum of its parts.

In 2012, the NSF funded two efforts: (1) Improvisational Theater for Computing Scientists (Holmes, 2011) and (2) RCN interdisciplinary Communication Lab for Undergraduate Education (Alonzo, Gaff, Watson, 2011). PIs and Co-PIs from these efforts organized a professional meeting: Cultivating Ensembles in STEM Education and Research (CESTEMER).

This meeting explored the relationship between performance, science and science education across a broad range of STEM disciplines. Of the 43 participants, members of ASCB presented posters, talks, and workshops on innovations in classroom curricula and dynamics (Wick and Matthes, 2011; Grabel et. al.

2013), building community and inclusion, facilitating improved mentor-mentee communication and basic biological research.

The interdisciplinary gathering inspired new work and collaborations within and beyond the life sciences that have led to publications, conference presentations, and performance based workshops to advance communication and collaboration skills. These performance-based approaches to STEM education are being integrated into undergraduate, graduate and professional curricula and courses at Boston University, Harvard Medical School, University of Washington and Rollins College. The goals include increased engagement with, understanding of and ability to communicate science among all audiences.

In this work, we present 1) how performance is used across these institutions to foster inclusion, collaboration and improved communication skills among scientists, faculty and students, and 2) how these scientists are continuing to organize opportunities to cultivate ensembles in STEM education and research.

P791

Board Number: B104

Biomedical Science Ph.D. Career Development and Interest Patterns by Race/Ethnicity and Gender.

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Recent biomedical workforce policy efforts have centered on the twin challenges of enhancing career preparation for graduate students and postdocs, and increasing diversity in the research workforce & professoriate. We report results from a survey of 1500 recent, American biomedical sciences Ph.D. graduates (including 276 from underrepresented minority (URM) backgrounds) that examined career preference changes over time, graduate training experiences, and determinants of career interest. At Ph.D. entry, 57% of respondents indicated their intentions to pursue a faculty career (a number that dropped to 42% by Ph.D. completion), yet fewer 25% indicated they entered graduate training knowledgeable about of the potential career options available to a Ph.D. biomedical scientist. Additionally, only 26% of respondents indicated they received structured career development during their graduate training. Scientists from all social backgrounds reported significantly decreased interest in faculty careers at research universities, and significantly increased interest in non-research careers (e.g. policy, law, consulting, etc.) at Ph.D. completion relative to entry. However, group differences by race/ethnicity and gender emerged in overall levels of interest (at Ph.D. entry and completion), and magnitude of the change in interest in these careers. Multiple logistic regression showed that when controlling for career pathway interest at Ph.D. entry, first-author publication rate, research self-efficacy, faculty support, and graduate training experiences, differences in career pathway interest between social identity groups persisted. All groups were less likely than men from well-represented (WR) racial/ethnic backgrounds to report high interest in faculty careers at research-intensive

universities (URM men: OR 0.60, 95% CI: 0.36-0.98, $p=0.04$; WR women: OR: 0.64, 95% CI: 0.47-0.89, $p=0.008$; URM women: OR: 0.46, 0.30-0.71, p

P792

Board Number: B105

Impacts of a supplemental course that emphasizes study skills for an introductory biology course: An analysis of exam performance and student perceptions.

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Objective: A supplemental course was designed to emphasize learning strategies to benefit students in an introductory biology course. *Methods:* Impacts of this course on exam performance were assessed by comparing the grades with students who did not take the class. This analysis was conducted over 4 consecutive semesters (178 students), examining C/D/F grades between the control and experimental groups. Additionally, students in the supplemental course were interviewed to identify common themes in regard to challenges perceived in the introductory biology class, attributes of the supplemental course that were useful to overcome the challenges, and to determine if there were any changes in study habits. *Results:* In regard to exam performance over the 4 semesters, the mean grades (C/D/F) differed significantly for the first exam (Control: 64.5; Exp: 61.5; p

P793

Board Number: B106

Modifying multi-section introductory labs to include student-designed experiments.

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Lab courses provide an opportunity to learn key scientific skills such as developing hypotheses, designing experiments with appropriate controls, analysis of results, and communication of findings. In order to introduce these skills into two sequential multi-section introductory biology lab courses, the “cookbook” versions of these courses were modified to include less breadth and more experimental design, as we have migrated structured lab protocols into student-designed experiments. One challenge with this approach is the inability to cover all topics within a semester. However, by constraining ourselves to a limited number of topics, we were able to emphasize elements of the scientific method in greater depth. Over the past several semesters, we have learned through trial and error which strategies are most successful. Student assessment from the first semester indicated that students were given only limited guidance and insufficient incentive for selection of research questions, proper experimental design, or even what should be included in their lab reports. We also found that consideration of

logistical constraints of these large multi-section lab courses is a major concern. For example, each section is taught by a different graduate teaching assistant (TA), some of whom are new to teaching, which can lead to major discrepancies between sections. Another concern is how to constrain student-designed experiments to the equipment, materials, and budget for these large multi-section courses. To address these concerns we have made a number of modifications that we are implementing this semester. Effective assignments for generating interesting research questions, increased student mentoring for experimental design, and clearer expectations for communicating experimental results have been developed. New TAs now attend a departmental workshop and weekly meetings aimed at giving them the skills necessary to help guide students through the process of experimental design. Student assessment of the lab courses will be presented, along with our syllabi, sample lab chapters and assignments from units emphasizing student-designed experiments, and our TA training agenda.

P794

Board Number: B107

Introducing computational scientific thinking materials into freshman biology curriculum.

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We present here a model for the incorporation of computational scientific thinking methods into undergraduate biology education. We evaluate if students develop an understanding and appreciation of the role of mathematical modeling and computation in scientific inquiry. In collaboration with Chemistry and Mathematics professors at Simmons undergraduate college, we pilot two computational science modules to first-year undergraduates. The modules provide practical computation approaches integrated into the scientific problem-solving paradigm. The materials will be introduced in October 2014 as part of the freshman learning community, *Your Microbes and You*. This course is an interdisciplinary problem-based class that uses bioinformatics to analyze the Human Microbiome.

Students learn to create models and perform simulations using Excel and Python programming language, which is used in other aspects of the course also. Our modules start with a scientific problem and then lead the students through its solution via building a computational model. We focus on exponential and logistic models of biological population growth. The *Exponential* and *Logistic Growth* modules are part of a larger set of curricular resources for teaching computational methods in scientific inquiry. These modules were developed for the NSF DUE project INSTANCES (Landau et al 2013, www.physics.orst.edu/~rubin/INSTANCES). Each module includes: learning objectives/skills/activities, a scientific problem, concept maps and system statements, the computational model, background information on the computational model, simulations of the model, and assessment in the form of formative assignments and a small project. Upon completion of the modules, we expect that students will gain a basic understanding and appreciation of the role of computation in biology.

We present the results of a survey evaluating the following outcomes:

- students' understanding of importance of computational methods in solving biological problems
- students' preference for a simulation tool: Excel or Python
- students' understanding of a process of creating computational model of biological phenomenon
- how students learn content introduced in combination with computational approach – if modeling enhanced understanding of biological question.

P795

Board Number: B108

A Biotechnology Techniques Course Investigating Genetic Modification of Local Foods Using Protein and DNA Analyses.

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Biotechnology Laboratory (BIOL4300) is an upper level course that focuses on biochemical and molecular biological laboratory techniques used in the field of Biotechnology. As part of the Georgia Gwinnett College 4-year Undergraduate Research Experience program, an initiative to give STEM students the opportunity to conduct course-embedded research at every stage in their undergraduate careers, the BIOL4300 curriculum has been redesigned to include an inquiry-based, semester-long project investigating genetically-modified (GM) foods. During the first phase of the project, students acquired skills needed to analyze foods at both the DNA level (DNA gel electrophoresis, traditional PCR, and Real Time PCR) and the protein level (ELISA, SDS PAGE, and immunoblotting). During the second phase, student groups were responsible for designing and conducting experiments to test food products from their local environments for the presence of GM food markers, including genes coding for *B. thuringiensis* crystal proteins and the glyphosate (“Round Up”)-resistant EPSP enzyme. Test foods included produce from local farmers’ markets, items from campus vending machines, and baby food sold at nearby grocery stores. Pre- and post-content assessment quizzes indicated that participation in the GM foods project increased student knowledge of biotechnological laboratory techniques and understanding of gene expression. Additionally, attitudinal surveys demonstrated that the new curriculum increased student interest in attending graduate school and pursuing careers in biotechnological research as well as student confidence in their ability to conduct independent research. Here, we present our curricular design and assessment as a model for incorporating the use of DNA and protein analytical techniques into a course-embedded, authentic biotechnological research experience.

P796**Board Number: B109****Data Analysis in the Biological Sciences: A 10-week hands-on course to equip students with tools for quantitative cell biology.**J.S. Bois¹;¹Division of Biology and Biological Engineering, California Institute of Technology, Pasadena, CA

The big questions in cell biology increasingly require quantitative approaches to get answers. A typical graduate student spends nearly as much time analyzing her data as she does acquiring them. Most graduate students, and indeed even senior researchers, learn analysis techniques as they need them. This often results in choosing the most familiar or commonly used analysis tool, as opposed to the optimal one.

To meet the growing needs of students as they begin their careers in quantitative biology, I developed a hands-on course covering basic data analysis techniques useful in cell and developmental biology. The goal is build a repertoire of *functional* skills with a basic understanding of the underlying mathematics. The target audience is senior undergraduates and beginning graduate students of biology and bioengineering. Each of the ten weeks of the course consists of a one hour lecture covering the theoretical background for the topic of the week, and a three hour "lab" session in which the TAs and I work with the students to analyze real biological data sets, including published results from literature and unpublished results from labs on campus. Each week's homework also features analysis of real data. Topics include regression, parameter estimation, outlier detection and correction, error estimation, denoising, hypothesis testing, image processing and quantification, and data display and presentation. (Absent is analysis of sequence data, for which Caltech offers a separate bioinformatics course.) We approach statistics from a Bayesian perspective and use Python as the instructional programming language.

I will present some of the examples of data sets we analyzed in the course, discuss the effectiveness of the course structure, and provide student feedback.

P797**Board Number: B110****3D printing in the biology classroom and laboratory: making the virtual tangible.**J.E. Honts¹;¹Biology, Drake University, Des Moines, IA

3D printing has existed for several decades, but the expiration of several key patents has promoted the rapid growth of the desktop 3D printing industry. A large array of affordable desktop 3D printers have become available in the last few years, ranging from DIY kits to ready-to-use pre-assembled printers. As a result, these printers had found increasing use in classroom, especially as a means of facilitating STEM

education. This technology provides faculty and students with the ability to turn virtual models of biological objects, whether organs, cells, or molecules, into tangible representations. A third generation MakerBot 3D printer has been used to explore how this technology can be used in the classroom and in the research laboratory. A diverse set of 3D printed objects have been fabricated for use in the lecture and laboratory class activities, for use as physical illustrations in research presentations, and for use as hands-on materials in outreach activities. This printer has also been used in research to build 3D representations of the distribution of GFP-tagged proteins in cells, using research data derived by means of confocal laser scanning microscopy. Computer-aided design and 3D modeling software packages have been used to precisely fabricate tools for use in teaching and research laboratories. A key accessory for a 3D printer is a 3D scanner, and we have adapted an Xbox console to capture 3D data from physical objects, which can be subsequently edited and printed. There are relatively few resources describing how to convert 3D data into appropriate data formats for 3D printing. Several protocols have been developed to prepare raw data and 3D models for 3D printing, starting with data file formats familiar to biologists. An unheralded aspect of 3D printing at present is the high frequency of print failures, which can occur for a variety of reasons, ranging from failure of printer hardware components to design flaws in the model to be printed. Tools and strategies have been identified to either prevent or correct many common problems. Desktop 3D printing is still in its infancy, it is not always user-friendly, and printers require continual maintenance. Yet it is clear that 3D printers will become a ubiquitous technology within the next decade. It is important that students begin to gain familiarity with this technology and its limitations. As has been the case with personal computers and mobile communications devices, 3D scanning and printing technologies have the potential to transform biological education, as well as basic and applied life sciences.

P798

Board Number: B111

Student development of cell culture laboratory modules for new curricular offerings in biology and bioengineering at Rowan University.

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Students designed and tested laboratory modules for a new upper level curricular offering “Fundamentals in Cell Culture Methods” as part of an NSF-TUES grant to integrate biomaterial science in the engineering and biology curricula. Two student-led teams, consisting of biology and bioinformatics students with varying levels of experience with cell culture, designed experiments to optimize human embryonic kidney (HEK)-293 cell proliferation through manipulation of culture media and substrate material. Cell proliferation was tested in different growth media at varying concentrations of fetal bovine, calf and horse serum with 10% fetal bovine serum determined to be the best medium for optimal HEK-293 cell growth. Cell proliferation in different substrates including bovine fibronectin, collagen I, gelatin, poly-L-lysine, bovine serum albumin were compared, with gelatin supporting the highest overall cell densities. In addition to designing experiments, students developed teaching

materials for cell culture modules, including lab protocols, instructions for the use of laboratory equipment, and course tutorials emphasizing data analysis and experimental design. The five undergraduate biology and bioinformatics students were specifically selected to represent the skill and experience levels expected for students that will ultimately enroll in the newly developed course. They ranged in academic standing from rising sophomores to seniors, cell culture experience from none to extensive, and prior research experience from none to one year. The students incorporated their prior laboratory and course-work experience in the overall conduct of the experiments and the subsequent writing of teaching materials for the curriculum. Throughout the project, the students developed specific laboratory skills such as aseptic liquid transfer, medium and substrate preparation, counting cells with a hemacytometer, passaging and freezing cells, microscopy, and use of ImageJ. In addition, students developed problem solving strategies, analytical skills, and curriculum development abilities. Students will continue module development and turn their focus to cell viability assays and cellular differentiation of mesenchymal stem cells, to further integrate bioengineering and cell culture concepts and skills in an upper level undergraduate lab-intensive course.

P799

Board Number: B112

Flipped Fridays in Cell Biology.

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In the Fall of 2013, I flipped one day a week of my Cellular Biology course. For the flip, students were required to watch 2-3 short video lectures and complete an online quiz on the content before class. Class time was used for students to work in groups on a problem set. The purpose of doing the flip was to give the students practice with challenging questions that asked them to apply what they had learned and interpret representations of experimental data. Comparison of the flipped Friday class with the previous semester, which was traditional lecture three days a week, showed no difference in exam averages between semesters. Analysis of student video viewing patterns using Kaltura metrics showed that videos needed to be fifteen minutes or shorter, and that most students were watching the videos only once on Thursday nights. A few students did not watch any videos and many students viewed some but not all, or started watching the video but did not finish. Student comments on evaluations indicate that most students liked the flipped Friday approach, but there is also student resistance to the method. Benefits of the flipped Friday were increased student-student interaction, increased faculty-student interaction, and introduction of more challenging problems. Ideas to improve student engagement further will be discussed.

P800**Board Number: B113****Introducing CourseSource: a peer-reviewed, open-access journal of evidence-based resources for teaching biology.**R.L. Wright¹, J. Blum¹;¹Biology Teaching and Learning, University of Minnesota, Saint Paul, MN

Over the past 20 years, many national reports have called for transformation of undergraduate science education. For example, in 2012, the President's Council of Advisors on Science and Technology issued the Engaged to Excel report, advocating implementation of evidence-based pedagogy to enable the United States to produce an additional 1,000,000 STEM graduates over the next decade. In response to such calls to action, colleges and universities nationwide are approaching their undergraduate STEM courses in new ways, but this change has been slow and fragmented. One of the greatest challenges to STEM education transformation is the time, energy, and vision that instructors need to create evidence-based lessons, courses, and curricula. Addressing this need was highlighted in the 2009 report, Vision and Change in Undergraduate Biology Education, which recommended creation of a repository of "student-centered, outcomes-oriented" resources. This poster will describe creation of a repository in the form of a peer-reviewed, open-access journal called *CourseSource*.

CourseSource publishes activities, lessons, labs, and other teaching resources that: (1) incorporate student-centered, evidence based pedagogy; (2) focus on professional society-developed learning goals and objectives; (3) can be quickly retrieval by instructors; and (4) are organized to enable easy implementation by other instructors. Publication in *CourseSource* provides authors with a high-quality vehicle for documenting their scholarly teaching efforts, accomplishments, and innovations.

To date, *CourseSource* includes learning objective frameworks for cell biology from ASCB, genetics from GSA, plant biology from ASPB and BSA, and microbiology from ASM. We expect that the appropriate societies will shortly approve frameworks for developmental biology, ecology, evolution, bioinformatics, biochemistry, and molecular biology. The site will go live to the public by the end of 2014.

Funded by a grant from the Howard Hughes Medical Institute

P801**Board Number: B114****RolePlaying: An Effective Pedagogical Tool.**L. Jayant¹, C. Priano¹;¹Science, Borough of Manhattan Community College, New York, NY

Engaging the interests of students in introductory science courses is a common challenge shared by educators all around the world. Our studies indicate that roleplaying can be used as an effective pedagogical technique to teach difficult concepts in undergraduate introductory majors Biology. Simple

roleplaying lesson plans can be designed to be quick while engaging a large number of students and without major effort or dramatic training. After explaining a cellular concept such as chemiosmosis during a regular lecture class, students are asked to act out the process. Students assume the roles of different molecules or structures. They then orchestrate a visual demonstration of the biological activity. The remaining students in the class participate as they critique, correct, and comment on their peers' acting roles. After the exercise, students are assessed on their knowledge and application of these concepts through quizzes or written assignments. We have successfully used roleplaying to reinforce concepts of chemiosmosis, mitosis, DNA replication, and protein synthesis. Our assessment results suggest that employing this technique as a supplement to conventional teaching methods greatly improves classroom involvement and retention of subject matter. Results of students' performance and retention rates will be discussed.

P802

Board Number: B115

Effects of in-class group problem solving sessions on group studying outside of class.

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Due to the demonstrated benefits of collaborative learning, we encourage students in our large biochemistry lecture course to study in groups. However, our anecdotal observations indicated that students use traditional study methods instead of more active methods, including group studying. We hypothesized that in-class group problem solving would increase the use of and/or improve attitudes about group studying. In 2013 we implemented two in-class problem sessions with 265 students randomly assigned to groups of 5. We evaluated studying behavior and attitudes using a study skills survey given to students at the beginning (pre) and end of the semester (post) and to alumni (2012) with no in-class problem sessions. Although student response to the in-class problem sessions was positive, the sessions did not significantly affect short-term behavior or attitudes on group studying. Interestingly, a number of students report feeling that group studying is effective, but do not use it regularly. We plan to investigate the reasons these students do not study in groups. In Fall 2014, the study skills surveys will be repeated and the problem sets will be implemented earlier in the semester. Our results will inform efforts to revise this course to encourage collaborative learning inside and outside of class.

P803**Board Number: B116****Does a first-year mentoring and research experience impact STEM retention?.**L. Caslake¹, C. Liew², R.A. Kurt¹;¹Biology, Lafayette College, Easton, PA, ²Computer Science, Lafayette College, Easton, PA

The Science Horizons program at Lafayette College involves 20 incoming first-year students in five mentoring sessions each semester, a three-week research project, and participation at a regional science conference. The mentoring sessions provide guidance concerning the many possibilities available to students on and off campus during their college years as well as career opportunities and graduate education directly or indirectly involving the life sciences. All 20 students participate in a three-week research project with faculty over our January interim session, a time when they can focus on research without commitments to classes. We anticipate that the mentoring sessions and early research experience will increase retention in the sciences and spark the interest of students in conducting more research. Over the past five years, 58% of students completing General Biology declared a STEM major, while over 90% of our first cohort declared a STEM major. Following their first year, five of these students are selected to receive support for three summers of research. Research projects in the first summer span multiple disciplines and require two research mentors. Following their second year, students conduct research on campus and following their third year, off campus. In addition to the regional conference, funding is available to send these five students to a national scientific conference of their choice. We hope to learn from students participating in these additional research experiences whether longevity in research impacts student retention and pursuit of advanced degrees. Early results show that the mentoring and early research experiences improve students understanding of the nature of science and research and provides a foundation for future studies for students interested in majoring in any STEM field. Students are asked to rate their skills in numerous areas in the fall of their first semester and are provided the same survey in the spring. The mentoring and early research experience allows student to recalibrate their self-assessment in numerous areas relating to general research skills and career options in the sciences. For example, in the fall students rated their general research skills as 'moderate to high' (4.3 on a 6 point scale), while after the interim research experience their retrospective analysis of their skills on entry dropped to 'very low to low' (2.5 on a 6 point scale; p

P804**Board Number: B117****Undergraduate research in the life sciences: Influence of the mentor.**M. Aikens¹, E.L. Dolan¹;¹Univ Texas-Austin, Austin, TX

Many undergraduate researchers in the life sciences are mentored by graduate or postdoctoral researchers (i.e., postgraduates) who themselves are mentored by faculty members, creating a

mentoring “triad” configuration. This unique mentoring configuration has not been the focus of empirical research even though faculty and postgraduate mentors are likely to have unique and important effects on undergraduate protégés. The goal of this study is to understand how undergraduate researchers in the life sciences are affected by being mentored by a postgraduate and a faculty member. Specifically, we are (1) empirically characterizing the range of mentoring triad structures at research universities, (2) determining how different mentoring triad structures relate to outcomes experienced by undergraduate researchers, and (3) determining whether female or underrepresented students experience different mentoring configurations and outcomes.

To accomplish this, we are collecting data from a national sample of matched triads: undergraduates and their postgraduate and faculty mentors. Using online surveys comprised of established, valid, and reliable measures, we are determining the types of triads found in undergraduate research mentoring, measuring the strength and functions of these mentoring relationships, and measuring the outcomes undergraduates realize from participating in research.

In the data collected to date, we have observed variance in mentoring triad structures, the functions of mentoring relationships, and the outcomes by triad type, demonstrating that students experience a range of mentoring. We have found that two triads are common in undergraduate research settings at research universities: a closed triad in which all members are connected, and a triad in which the undergraduate interacts with the postgraduate and the postgraduate interacts with the faculty member. Our preliminary analysis indicates that undergraduates in closed triads reported greater outcomes related to learning, confidence in their scientific abilities, and scientific identity than undergraduates in triads with only undergraduate-postgraduate and postgraduate-faculty ties. Therefore, mentoring structures in which the undergraduate directly interacts with both the faculty member and the postgraduate appear to be more beneficial than those in which the undergraduate is only interacting with the postgraduate. This is supported by the additional finding that postgraduates and faculty differ in the support they give; postgraduates provide more instrumental support (i.e., “how-to” help) whereas faculty provide more networking support.

For undergraduate research programs to be effective, we need to understand the ways in which mentoring affects undergraduate outcomes. The results of this work will yield unprecedented insights into the latitude we have for designing undergraduate research experiences.

P805**Board Number: B118****Spreading Vision and Change Through Long-Term Mentorship Opportunities: the ASCB Mentorship in Active Learning and Teaching (MALT) program.**M.J. Wolyniak¹, D.E. Allen², J.K. Hood-DeGrenier³, G.E. Plopper⁴, A.J. Prunuske⁵, S.M. Wick⁶, A.N. Becalska⁷, J. Dyer⁸, L.A. Gurski⁹, M. Kokes¹⁰, S. Murugesan¹¹, W. Xie¹²;¹Biology Department, Hampden-Sydney College, Hampden-Sydney, VA, ²University of Delaware, Newark, DE, ³Worcester State University, Wellesley, MA, ⁴Rensselaer Polytech Inst, Troy, NY, ⁵Medicine, University of Minnesota-Duluth, Duluth, MN, ⁶University of Minnesota, Saint Paul, MN, ⁷Biology, Brandeis University, Waltham, MA, ⁸MIT, Cambridge, MA, ⁹Urology, Univ Pittsburgh Med School, Pittsburgh, PA, ¹⁰Biology, Duke University, Durham, NC, ¹¹NIH\NHLBI, Bethesda, MD, ¹²Institute of Medical Biology, Singapore, Singapore

Extensive pedagogical research recognizes active learning strategies as the most effective way to teach STEM disciplines, yet a disparity exists between these ideas and their widespread and effective practice. To promote reform in undergraduate STEM education, the 2013 *Vision and Change* in Biology Undergraduate Education working group challenged professional scientific societies to take action to help their members implement lasting changes in their approaches to teaching. In response to this call to action, the ASCB has created the Mentoring in Active Learning and Teaching (MALT) program, which is designed to give society members access to a long-term hands-on experience implementing active learning in their classrooms. This small grant program allows individuals at any point in their career path to work with faculty who are experienced in employing active learning practices in their classrooms, providing mentees with hands-on experience and long-term mentorship in active learning best practices. A pilot version of MALT began in late 2013, matching 6 experienced active learning-based teachers with enthusiastic mentees responding to a call put out in ASCB communications. The mentees have thus far engaged in a range of activities with their mentors: giving department seminars; collaborating on course development; and developing multi-session active-learning modules. Preliminary assessment through interviews with mentees suggests a rise in their confidence in developing and implementing active learning strategies in their classes. As the program grows, we intend to assess the degree of use of active learning materials with a newly developed, validated tool called the Classroom Observation Protocol for Undergraduate STEM (COPUS) (Smith *et al.*, 2013). COPUS utilizes a coding system to observe and record the actions of instructors and students at given points of time in a class period. The ASCB intends to partner with the Genetics Society of America (GSA) and the American Society of Plant Biologists (ASPB) to create an expanded version of MALT that will best serve the increased need for active learning mentorship.

P806**Board Number: B119****Genome Solver: Training Faculty to Deliver Bioinformatics Content to Life Science Students.**A.G. Rosenwald¹, G. Arora¹, R. Madupu², J.A. Roecklein-Canfield³, J. Russell⁴;¹Department of Biology, Georgetown University, Washington, DC, ²J. Craig Venter Institute, Rockville, MD, ³Department of Chemistry, Simmons College, Boston, MA, ⁴Center for New Designs in Learning and Scholarship, Georgetown University, Washington, DC

Bioinformatics is becoming a routine part of biological research, yet students entering the workforce may not have had much training in this relatively new discipline. This stems in part from the fact that many life science faculty have little training and so are reluctant to teach this material. To address this deficit, we have developed the Genome Solver Project. Over the past 3 years, we have trained more than 120 faculty members at a variety of different institutions, including community colleges and minority serving institutions, to use basic, open-source bioinformatics tools, within a context involving good pedagogical practices. In addition, we are now in the process of developing a community science project using the rich DNA sequence data sources available in public databases to examine horizontal gene transfer between bacteriophages and bacteria to demonstrate how to use bioinformatics for original undergraduate research experiences.

P807**Board Number: B120****Biology of Global Health: Preparing Students for Multi-disciplinary Group Work.**H.G. Elmendorf¹, A.G. Rosenwald²;¹Georgetown University, Washington, DC, ²Department of Biology, Georgetown University, Washington, DC

Training undergraduate students to tackle complex multi-dimensional issues is a challenge for educators. Problems that will have relevance into the future include ones in global health – chronic and infectious disease, clean water and food for 7 billion people, pollution, climate change, etc. Issues like these require multidisciplinary teams with expertise in different realms, and the experts need to be able to communicate with one another to bring about effective interventions. In order to address this challenge, we have developed a novel major, called Biology of Global Health (BGH). This major emphasizes science (students take at least 10 courses in biology and at least 6 additional courses in chemistry, math, and statistics), but also emphasizes the interplay between science and society (students are also required to take at least 2 courses in ethics, policy, economics, international health, history, etc. beyond the required college core requirements).

Much of class time in the sophomore level Introduction to BGH course, the gateway to the major, is devoted to discussing information from a wide variety of sources, including the scientific literature.

However, much of the work occurs outside of class, where students in teams of 3-5 tackle projects in which they must communicate a scientific topic to a particular audience using a specific type of media. Recent projects have included a website describing a health disparity in the District of Columbia to members of the community, a policy briefing to a member of the Institute of Medicine on tobacco use, a 1 minute video public service announcement for use by a local food bank, and the specific aims page for an NIH grant, among others. Reflections from the students after each project uniformly mention that given the short turn-around time for each project (usually 2 – 2 ½ weeks), teamwork was crucial for creation of an effective final product. Survey results from graduates of the major show that learning how to work in groups is an important skill they carried forward. Therefore the Introduction to BGH course gives students a chance to practice teamwork skills that will be useful in their post-graduate careers, skills that are often absent in many lecture-based science classes.

P808

Board Number: B121

Incorporation of linked research experiences into biology and chemistry courses promotes student confidence and interdisciplinarity.

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Pedagogical studies suggest that course-based undergraduate research experiences (CUREs) can increase student knowledge of and interest in related subject matter, improve student retention, and influence student choice of academic and career paths. Explicitly interdisciplinary curricula also positively impact students by increasing awareness of the connections between scientific disciplines and improving articulation of cross-disciplinary concepts. Here we describe the development of cross-disciplinary CUREs that are integrated into upper and lower-level biology and chemistry courses. Students in impacted labs develop their own hypotheses and apply a variety of biological and chemical approaches to make novel discoveries about the anticancer ruthenium complex indazolium *trans*-[tetrachlorobis(1*H*-indazole)ruthenate (III)], also known as KP1019. To promote cross-disciplinary thinking and collaboration, students upload their findings to a CURE-associated wiki and consult the wiki during experimental design and data analysis. On anonymous end-of-semester surveys, students self-reported that these CUREs significantly increased their degree of experience with integrating disciplines. Since students constructed large portions of the wiki, its structure was analyzed as a possible measure of students' cross-disciplinary thinking. The large proportion of links between data generated in chemistry and biology courses supports the conclusion that these KP1019-focused CUREs promote student appreciation of interdisciplinarity. These results are consistent with the findings from focus groups conducted by an external evaluator. This consultant also noted that students who participated in more than one KP1019-focused CURE reported greater learning gains. In addition to the unique benefits associated with these cross-disciplinary CUREs, formative assessment data suggest that the gains associated with more traditional CUREs were still attained. For example, students self-reported

significant increases in their degree of experience with working on problems with no known outcome. With respect to their ability to interpret results, to analyze data, and to write scientifically, students reported gains comparable to those obtained during intensive summer research programs. In addition to validating the pedagogical value of integrating research and teaching, this work suggests that cross-disciplinary CUREs provide a robust mechanism for helping students integrate their knowledge of different disciplines. Moreover, virtual collaboration tools, such as wikis, can be used to illustrate the interdisciplinary nature of modern research.

P809

Board Number: B122

Research Experience for Peruvian Undergraduates (REPU): A sustainable student-run scientific training program for STEM undergraduates in developing countries.

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The Research Experience for Peruvian Undergraduates (REPU) program is a science outreach effort that aims to build scientific capacity in Peru. The program complements Peruvian undergraduate scientific education by setting up 10-week research-intensive internships in laboratories in the United States and Europe in the biomedical sciences, chemistry, and engineering. Peruvian graduate students and post-docs all over the world have run the program since 2007. So far, 25 outstanding Peruvian undergraduates have participated in the three branches of the program: REPU-Biology, REPU-Chemistry and nanoREPU.

Over the years, the program structure has evolved empirically: the program has implemented several educational techniques to enhance the scientific experience of the undergraduate participants. The program relies on a multi-step and rigorous selection process, and a one-on-one mentor-mentee strategy. Our selection process ensures an outstanding student performance in their host laboratory; for instance, 6 students have stayed as post-bac associates, and 2 publications have REPU alumni as authors for their contributions during the internship. In our mentor-mentee strategy, Peruvian graduate students mentor REPU students and help them shape and pursue their academic goals. Our methodology have allowed REPU alumni that applied to graduate school to have a 100% acceptance rate at competitive graduate programs at Yale University, Harvard University, Stanford University, etc. Finally, after being part of the experience, around 80% of our alumni have taken leadership roles in at least one of the organizational committees of the program, and two have become directors of the program, making the program sustainable. We believe the REPU model can be reproduced to be successful in other developing countries sharing Peru's scientific situation.

The educational model that REPU currently uses is dynamic, sustainable, and successful. In the next few years, REPU plans to expand to new branches of science like physics, and to new locations like Canada. Thus, REPU will continue pursuing its main goal: to help building scientific capacity in Peru by creating a long-lasting network of young, well-connected scientists who are committed to helping Peruvian science.

P810

Board Number: B123

Creating an authentic research experience in an undergraduate advanced cell biology laboratory.

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Research and research-like course activities have been shown to enhance the educational experience of undergraduate students. The goal of this project was to incorporate bona-fide research into a course-associated, undergraduate advanced cell biology laboratory. Advanced Cell Biology is a 400-level course with lecture and laboratory components that enrolls 12-16 students. The goals of the laboratory were to engage students in an authentic research project, yielding novel data, and to facilitate implementation of the scientific method. Each student pair was assigned a strain of fission yeast that contained a deletion in a gene of interest. Genes were selected based on the following criteria: 1) the gene had never been characterized in fission yeast, and 2) analysis of homologs revealed a potential function in membrane transport. During the first week of lab, students used freely available bioinformatic tools (<http://workbench.sdsc.edu/>) to analyze their genes and identify homologs in other species as a basis for literature searches. Over the next 7-8 weeks, students performed instructor-designed assays to compare cell growth and division, drug sensitivity, and membrane trafficking between the wildtype and mutant strains. Based on findings from this structured portion of the laboratory and the literature, students then developed a hypothesis regarding the function of their gene and designed an experimental procedure to test their hypothesis that was carried out in the final 2-3 weeks of the laboratory. The most surprising barrier to implementing the laboratory was student expectations. Students were initially uncomfortable with not knowing what “should” happen. Encouraging discussion and data comparisons between different groups seemed to alleviate student anxiety. Students were evaluated based on laboratory participation, a laboratory report, and a final presentation describing the hypothesis and data from their individual projects. Evaluations showed that 87.5% of students rated the laboratory as excellent with respect to “overall evaluation of this laboratory experience in teaching the discipline and its techniques.” Comments revealed that “the labs never seemed like a waste of time because every experiment had a new goal and new results.” Students found that the lab “[got] students excited/involved in research” and “challenged students to interpret results.” The class also promoted further interest and engagement in scientific research for juniors. The lab was beneficial for the instructor’s research laboratory, as preliminary findings led to further studies that resulted in a

publication. This type of research-like experience is being implemented in an increasing number of laboratories at BSC, and we hope to engage more and younger students.

P811

Board Number: B124

The effects of herbal remedies in reversing chemically induced cell death: an undergraduate project encompassing cell biology, molecular genetics, and neuroscience.

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Cell biology is at the nexus of many biological subdisciplines, including molecular genetics, developmental biology, and neuroscience. The project described here brings together these areas of research as undergraduate biology, neuroscience, and genetic engineering majors investigate the effects of teratogens, apoptosis-inducing chemicals, and herbal supplements on cell growth, ion channel physiology, and gene expression. The faculty partnership has spanned five years, beginning as a project that bridged upper-division courses with the objectives of increasing content knowledge across subdisciplines and providing research experiences in teaching laboratories. This project has since been expanded to include students working towards their undergraduate theses as well as summer interns, and portions are supported by an NSF-TUES grant. The initial project investigated gross morphology of treated chicken embryos or isolated chick neurons in combination with a microarray analysis of gene expression across several thousand genes. To better examine cellular apoptosis, the project has shifted to cell culture models, mainly focusing on DT40 chicken lymphoblast cells and using HeLa cultures for comparisons. Genetic analysis has progressed to the use of pathway specific PCR arrays and real time PCR analysis. Furthermore, electrophysiology has been incorporated through the use of an automated patch clamp system that is pedagogically amenable to undergraduate use. Challenges encountered in this project have ranged from ones typically noted in undergraduate laboratories, such as bacterial contamination of cells, to much more sophisticated research dilemmas. An example of the latter was the decision to choose a non-neural, immortal cell line to replace primary neuron culturing, to allow for a project focused on a single cell type rather than a mixed culture of neurons, glial cells, and fibroblasts. An additional challenge is that this cell line has been described in the literature as lacking p53, leading to questions about the activation of apoptosis pathways in these cells; measuring apoptosis using fluorescent markers has been difficult to replicate and quantitate. Future experiments will focus on treatment and assessment of additional cell lines, such as the human neuroblastoma SH-SY5Y, and on purified chicken neurons. One outcome of this project has been student-developed and student-revised protocols that are written explicitly for use by undergraduates. Student learning gains and shifts in attitudes toward scientific research were assessed before and after participation. In addition, student researchers have collected cell images, quantified cell survival, and assayed gene expression successfully and will describe this work in student poster presentations.

P812**Board Number: B125****Caenorhabditis elegans as a Potential Model for Manganese Nanoparticle Toxicity.**T. Smith¹, F. Norflus¹, J. Jordan¹;¹Department of Natural Sciences, Clayton State University, Morrow, GA

As part of the Careers in Science Initiative at CSU to evolve freshman and sophomore students in undergraduate research, one of the authors spent the summer researching the dangers of exposure to metal oxide nanoparticles (less than 100 nm size), like manganese nanoparticles. The study involved using the nematode worm *Caenorhabditis elegans* (*C. elegans*) as the model organism. Our interest lies in the indication that nanoparticles such as Mn₂O₃ have been involved in the Parkinson's disease-like symptoms that are irreversible (manganism). To study the neurotoxicity of nanoparticles, we exposed *C. elegans* to increasing concentration of Mn nanoparticles. These worms are routinely used in the neurotoxicity of drugs and pesticides. In this study, we observed the morphology of the worms following a 1 and 5 day exposure to increasing concentration nanoparticles (up to 1000 µg) using light and fluorescence microscopy. Oxidative stress was monitored in the control and treated worms using a fluorescent dye marker. The results suggest an increase in the generation of free radicals following an increase in nanoparticle concentration. Preliminary studies also suggest a change in the gene expression of CED-9 gene in *C. elegans* and indicator of some protection of the worm from programmed cell death. In summary, *C. elegans* may serve as an important model in evaluating the toxicity of Mn nanoparticles.

P813**Board Number: B126****Careers in Science College Initiative: Undergraduate Research Experience for Freshman and Sophomore Biology and Chemistry students.**J. Jordan¹, F. Norflus¹, R. McFarlane¹, D. Day¹;¹Natural Sciences, Clayton State University, Morrow, GA

On numerous occasions, students enter college unprepared for the rigor of pursuing a degree in a science-related field. Studies show student involvement in undergraduate research early in their careers has a dramatic impact on academic success as well as providing personal and professional benefits for the student. The overall goal of this initiative was to stimulate student interest in careers in science by involving them in an undergraduate research experience with faculty. The close interaction with faculty would help the student develop important academic, research, and communication skills. These skills are carried over into the classroom setting and assist in building student self-esteem and confidence. Workshops were held where faculty gave short presentations of their research. The faculty also talked with the students about different opportunities for undergraduate research and what opportunities this

would afford them in the future. At a different informational meeting, the faculty gave presentations on maintaining a laboratory notebook, laboratory safety, and on how to do perform literature detailed literature searches. The students were recruited to the program by the faculty advertising this initiative in posting flyers around campus and announcements in introductory biology classes. In order to assess the effectiveness of this project, the students were given a pre-test prior to their involvement in the project and a post-test at the end. On these tests, the students were asked demographical data relating to their backgrounds, their knowledge of careers in science, and necessary skills in laboratory science. The end of the test then asked the students specific questions relating to the research of the faculty with whom they decided to work. Several students have participated in this project. To date, the data has been positive and supportive of the importance of involving students early in their scientific career in undergraduate research.

P814

Board Number: B127

Implementing a Classroom Undergraduate Research Experience (CURE) in a Bioinformatics and Genomics Class.

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Delivering upper-level undergraduate courses in bioinformatics and genomics at a Primarily Undergraduate Institution (PUI) presents unique challenges. A special difficulty is that course assignments and hands-on activities often become obsolete or outdated from one year to the next as online tools change so rapidly. BIOL323 "Bioinformatics and Genomics," an upper-level major elective at Lock Haven University, can only be offered every other year, compounding the difficulty. As a number of educators in the field have confronted these issues, we have sought to formalize our collaboration in order to develop and implement a Classroom Undergraduate Research Experience in Bioinformatics (CURE). To this end, in the summer of 2012 Lock Haven University joined the Genomics Education Partnership (GEP), a consortium of PUI faculty working in partnership with the Department of Biology and the Genome Institute at Washington University in St. Louis. The goal of the Partnership is to provide professional development, software tools, and an overarching project framework that allow faculty to provide publishable research projects in the context of undergraduate courses in bioinformatics and genomics. By distributing the work of annotation of genes and other genome features among a large number of undergraduates enrolled in courses at many institutions, the Partnership maximizes both research productivity and opportunities for up-to-date, hands-on, real-world experiences for participants, directly analyzing fundamental questions in the field. Classroom activities and their pedagogical and research outcomes will be presented.

P815**Board Number: B128****Identification and annotation of gene features in *Drosophila biarmipes* contig59 using a computational-genomics approach.**J. Nelson¹, T. Sadikot¹;¹Biology, Washburn University, Topeka, KS

The genome of *D. melanogaster* was completed in 2000 and it has since become a model organism. This fly is one of the most studied species in biology and serves as a model organism for studying many developmental and cellular processes common to higher eukaryotes. In this study, the well-annotated *D. melanogaster* was used as a reference for conservation-based analysis to annotate and identify all the genes present within the contig59 sequence of *Drosophila biarmipes*. Using a number of open-source computational genomic tools for sequence alignment, gene-prediction and *Drosophila* genome browsing, the *Drosophila biarmipes* contig59 was examined for relevant genomic elements such as genes, pseudogenes and repetitive elements. Contig59 corresponds to the dot chromosome of *D. biarmipes* and was observed to contain three putative genes, all in the order and minus orientation, consistent with that found in *D. melanogaster*. Our analysis revealed that of the three genes predicted within this contig, CaMKI erroneously appears in this region only due to shared-conserved domains with CamKII. Thus, in our final conclusion, we state that the *Drosophila biarmipes* contig59 only contains two genes, CamKII and Zyx which are both well conserved between *D. melanogaster* and *D. biarmipes*.

P816**Board Number: B129****The Genomics Education Partnership (GEP): An undergraduate bioinformatics research network providing transformative course-based research experiences.**M. Laakso¹, C. Arrigo², J. DiAngelo³, D. Dunbar⁴, C. Howell⁵, A. Kleinschmit⁶, L. Paliulis⁷, J. Porter-Kelley⁸, J.A. Roecklein-Canfield⁹, A.G. Rosenwald¹⁰, M.J. Wolyniak¹¹, L. Zhou¹², W. Leung¹³, C. Shaffer¹³, S. Elgin¹³;

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The *Vision and Change* and PCAST reports call for an increased use of collaboration and research-based educational practices as vital to engaging students in modern science and preparing them to be both

potential participants in the STEM workforce and informed citizens in a complex society. The use of bioinformatics in a multi-institutional community of practice provides an outstanding way for students at all types of institutions to engage in research resulting in real and publishable scientific data in an accessible and inexpensive manner. The Genomics Education Partnership (GEP) is a network of more than 100 colleges and universities serving over 1000 undergraduates annually that uses a central platform at Washington University in St. Louis to provide course-based research experiences that can be adapted to the specific needs of each class and institution type, including large universities, small undergraduate institutions, and community colleges. GEP research projects are used in short introductions (~10 hr), deeper class investigations (~ 44 hr), and independent study. GEP curriculum, which utilizes the power of comparative genomics, focuses on research questions considering the evolution of the Muller F element, a domain with unique hetero- and euchromatic properties, in multiple species of *Drosophila*. GEP undergraduates work to improve the draft genomic sequences and/or annotate these improved sequences. Their findings contribute to student co-authored publications, provide data for elucidating the regulation of chromatin structure and its impact on gene expression, and benefit the scientific community through data submission to public databases. We find that students who engage with GEP materials, regardless of institution type, show noteworthy learning gains as well as the ability to prosper in a research-intensive curriculum. Current work in the GEP is focusing on how to best adapt bioinformatics curricula for use in an introductory-level biology course, and how to synergize with wet-lab components. Overall, national projects like the GEP take advantage of modern technologies to build cost-effective collaborations that engage undergraduate classrooms; these strategies are having a widespread impact on life-science teaching.

P817

Board Number: B130

Maximizing Undergraduate Research Opportunities By Combining Independent Senior Projects With Lower-Level Classroom Labs.

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In recent years, a wide variety of institutions have implemented course-based research experiences (CUREs) as an integral part of a complete education in the life sciences. An inhibiting factor for many faculty interested in developing CUREs is the development of research questions that can be adequately expanded and adapted for a large number of students in a classroom setting and that also provides students with a feeling of project ownership. At Hampden-Sydney College, a small liberal arts college in central Virginia, we are taking advantage of senior honors projects to develop CUREs that can be employed in introductory or intermediate-level biology classes. We believe that this system of CURE development can provide a variety of authentic research experiences to the majority of Hampden-Sydney biology majors while also allowing an exciting level of undergraduate engagement in the process as upperclassmen play a significant role in the planning and execution of research that will be performed

by themselves as well as their fellow students. As a pilot for this CURE development model, we are using a project based on antibiotic design to combat bacterial infection. The project builds on the work of Wenzel *et al.* (2014) that tested the effectiveness of a peptide consisting of arginine and tryptophan repeats (RWRWRW-NH₂) as an antibiotic. This work found that the peptide could embed itself in the cell wall of a bacterium but left many questions unanswered as to the efficacy of derivatives of this peptide against common bacteria of genus *Bacillus* and *Staphylococcus*. A research project of this nature requires the synthesis of several polypeptide derivatives and the testing of these polypeptides against several bacterial species; thus, we believe it is a strong candidate to serve as both a senior honors project and a CURE that can be taught by a faculty instructor with the assistance of senior honors researchers.

We are using this antibiotics project during the 2014-15 academic year in Hampden-Sydney's Genetics and Cell Biology course, which is traditionally taken in the first two years of a student's undergraduate education. Students are taking polypeptides designed by the senior thesis researchers and using them to design and execute experiments on their ability to influence the ability of *Bacillus* and *Staphylococcus* growth. Assessment of the CURE is focused on student confidence in their ability to perform authentic scientific research, attitudes on project ownership, and communication of results in paper and poster forms. Thus, this CURE represents the development of an exciting pedagogical model in which upperclassman research projects provide research participation opportunities that can reach the majority of biology students.

P818

Board Number: B131

Amplification and RFLP analysis of the "caffeine gene" in the genetics classroom.

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Caffeine is the most popular stimulant in the world. However, the effect of caffeine on an individual varies widely. Along with environmental factors, this is due, in part, to the genetic differences in how caffeine is metabolized by liver enzyme cytochrome P450 1A2 (CYP1A2). Those who metabolize caffeine quickly will be less affected, whereas slow metabolizers will feel the effects long after consumption. A single nucleotide polymorphism (SNP rs762551) within an intron of CYP1A2 exists as either an A or a C. Fast metabolizers are homozygous A, while those who are homozygous C or heterozygous are slow metabolizers. The goal of this project was to generate a series of classroom laboratories analyzing this SNP from the students' own DNA. DNA was isolated from cheek cells, and a specific region of CYP1A2 was amplified via PCR. The PCR product then underwent RFLP analysis using the enzyme *Apal* to detect the SNP, and *SacI* served as a control. Using agarose gel electrophoresis, each genotype was distinguished by the size of the RFLPs. As additional controls, unknown data were compared against individuals reported to be homozygous A, homozygous C, or heterozygous by the DTC genetic testing company 23andme. Using RFLP is a more cost-effective analysis than other sequencing-based

laboratories. Importantly, this exercise can be scaled up or down to meet the needs of majors and non-majors alike. Overall, this series of laboratories exposes students to a variety of basic genetics techniques, correlates to an observable phenotype without causing serious health concerns, and caters to students' innate desire for self-discovery.

P819

Board Number: B132

pClone: A New Synthetic Biology Laboratory Module that Improves Student Learning.

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The Vision and Change report recommended genuine research experiences for undergraduate biology students. Authentic research improves science education, increases the number of scientifically literate citizens, and encourages students to pursue research. Synthetic Biology is a new, interdisciplinary field that blends chemistry, engineering, mathematical modeling, computer science, molecular biology and systems biology. Synthetic biology is well suited for undergraduate research, is practiced by a growing community of undergraduate educators, is attracting federal and corporate funding, and provides promising job opportunities for future scientists. After years of training undergraduates to conduct synthetic biology research, we are familiar with the best methods to use with inexperienced students. Our pedagogical goal was to develop an inexpensive and simple laboratory module that AP Biology and college faculty could adopt easily to their local conditions while accomplishing specific learning objectives. We developed a laboratory module called pClone that empowers students to use advances in molecular cloning methods to discover a new transcriptional promoter or mutate a canonical promoter and measure promoter activity in *E. coli*. pClone uses typical laboratory equipment, requires minimal preparation time and meets the increased demands placed on faculty and departments. pClone leverages the modern method of Golden Gate Assembly as a cutting-edge molecular cloning tool that can be used by students with little or no experience in molecular biology. We also developed the Registry of Functional Promoters, an open access database of student promoter research results. This poster describes our "action research" that documents learning gains by students using pClone and details the simple procedures by which pClone can be adopted by others. Action research is defined as faculty-led research and dissemination of an innovation that enhances student learning. Our educational goals are consistent with Vision and Change and emphasize core concepts and competencies. Using pre- and post-tests, we measured significant learning gains among students using pClone in Introductory Biology and Genetics classes. Student post-test scores were significantly better than scores by students who did not use pClone. pClone is an easy and affordable mechanism for large

enrollment labs to meet the high standards of Vision and Change. The pClone module provides an inexpensive entry point to synthetic biology and promoter analysis for faculty to offer authentic research experiences for their students within the context of laboratory classes.

P820

Board Number: B133

The “WHY, HOW, WHERE, WHAT, SO WHAT?” strategy for teaching scientific Results writing in undergraduate courses: what is it, and does it work?.

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Learning to write about data in the style of a scientific research paper is a valuable experience for undergraduate students regardless of whether they may ever be in the position of actually preparing a manuscript for publication. Asking students to write a standard Results section in particular provides a measure of a student’s understanding of experimental questions and procedures, as well as their ability to analyze data and draw logical conclusions. Furthermore, learning to craft a well-written Results section develops general organizational and communication skills that students can apply to other forms of writing and to oral presentations. Thus, there is clear value in pairing research paper-style writing assignments with course-associated laboratory investigations. However, students often find such assignments daunting, and instructors are often dissatisfied with the outcomes, especially given the effort required to evaluate and provide feedback on this type of student work. To address these problems, we have developed, implemented, and assessed the efficacy of a “formula” to assist students with writing a Results section: the “WHY, HOW, WHERE, WHAT, SO WHAT?” strategy. We will detail this teaching strategy, provide examples of assignment descriptions and grading rubrics that employ it, and present data from two types of assessment: student surveys and rubric-directed evaluation of student writing from introductory, intermediate, and upper level courses in cell and molecular biology and genetics.

P821

Board Number: B134

Using HeLa cell stress response to introduce first year students to the scientific method, laboratory techniques, primary literature, and scientific writing.

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Incorporating scientific literacy into inquiry driven research is one of the most effective mechanisms for developing an undergraduate student’s strength in writing. Additionally, discovery-based laboratories help develop students to approach science as critical thinkers. Thus, a three-week laboratory module for an introductory cell and molecular biology course that couples inquiry based experimental design with

extensive scientific writing was designed at Westminster College to expose first year students to these concepts early in their undergraduate career. In the module students used scientific literature to design and then implement an experiment on the effect of cellular stress on protein expression in HeLa cells. In parallel the students developed a research paper in the style of the undergraduate journal BIOS to report their results. HeLa cells were used to integrate the research experience with the Westminster College "Next Chapter" first year program, in which the students explored the historical relevance of HeLa cells from a sociological perspective through reading *The Immortal Life of Henrietta Lacks* by Rebecca Skloot. This work describes the design, delivery, student learning outcomes, and assessment of this module and while this exercise was designed for an introductory course at a small primarily undergraduate institution, suggestions for modifications at larger universities or for upper division courses are included. Finally, based on student outcomes suggestions are provided for improving the module to enhance the link between teaching students skills experimental design and execution with developing student skills in information literacy and writing.

P822

Board Number: B135

Crowd-funding for Undergraduate Research.

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Being an educator and conducting mentored research with students at a primarily undergraduate institution is both incredibly rewarding and challenging. The two main factors that play into a faculty's productivity are time and resources. It is difficult to maintain an active research program while teaching three or four classes every semester and trends in higher education have institutions and departments strained for resources. The increasing cost of education in our country, ever-changing labor markets, and the introduction of new technology have many universities facing serious financial struggles. As educators and undergraduate mentors we need to start getting creative about our time and funding capabilities. For many of us summer is the time when we can really focus on our research, but funding opportunities for summer projects are scarce and applying to larger governmental grants is time-consuming and very competitive. I turned my sights to crowd-funding. The two science-specific crowd-funding websites that I researched are Start Up Scholar and Experiment.com. These crowd-funding websites have a platform for you to design a website with images, videos, scientific explanations, a defined budget, and endorsements from the scientific community. It is then the responsibility of the researcher to advocate and spread the word about the campaign. The project is only funded if the monetary goal is reached within a defined time period. These professional websites then take a small percentage (5-7%) of your funds and provide you with a check for the remaining amount. My crowd-funding campaign on Experiment.com entitled "Flies on the Brain: Can Fruit Flies Aid in Curing Neurodegeneration?" was kicked off in April 2004 and 30 days later was successfully funded for \$4125. This funding covered housing and summer salary for a summer undergraduate research student conducting biomedically relevant research in my laboratory.

P823**Board Number: B136****Tactile Literacy: Customizing STEM Education.**A.N. Gonzales¹, L. Van Tuyl¹, B. Martinez¹, E. Hasper², T. Hedgpeth³, R.A. Windhorst², D.P. Baluch¹;¹School of Life Sciences, Arizona State Univ, Tempe, AZ, ²School of Space and Earth Exploration, Arizona State Univ, Tempe, AZ, ³Disability Resource Center, Arizona State Univ, Tempe, AZ

Achieving a successful career in STEM (science, technology, engineering and mathematics) requires not only a great education but extensive training and preparation. This high level of training is especially important with the predicted shortage of qualified professionals where it is estimated that by 2018 the nation is expected to have 8.6 million STEM jobs available where 60% will require skills possessed by only 20% of the workforce. Over the last decade, the U.S. government has invested \$3 billion dollars a year in 220 programs across 13 different agencies to improve the STEM education system in order to create a sustainable pipeline of trained professionals. Although many programs have been created to provide additional training to graduate and post-doctoral students to prepare their transition into a professional appointment, the focus is beginning to shift to the K-12 and post-secondary level education to ensure that students not only receive adequate training but are encouraged to pursue a STEM career. In a recent study, researchers developed customized tactile teaching tools, such as images converted into 3D tactile boards, to test if these could increase the performance and participation of visually impaired students. Results from this study found that both sighted and blind/visually impaired (BVI) students, who were taught using the hands-on tactile laboratory setting, enjoyed their lessons much more than their peers who only participated in the text based format. When compared on retention and understanding, the tactile learners received an average increase of 15% in their test scores as compared to their peers who used the text based format. These assessments demonstrated that in order to improve student performance, some concepts, especially in lab based disciplines, are best taught using a tactile learning style that moves beyond what standard Braille and raised line diagrams, graphs and outline drawings are able to provide. An additional benefit of this study was that the tactile teaching elements, which are customizable to the subject area, enable instructors to reach a greater diversity of multidisciplinary students. Results from this study suggest that tactile literacy may become viewed as a necessary component to help sighted and BVI students pursue higher levels of post-secondary education and ultimately increase the prospect of obtaining professional employment in a STEM field. In addition, these findings highlight the importance of developing and testing new customizable tactile technologies in order to make STEM education accessible and enjoyable for future students.

P824**Board Number: B137****Development and Assessment of an Undergraduate Teaching Assistantship (UTA) Program in Biology Laboratories at American University.**N. Zeller¹, M. Bentley²;¹Biology, American University, Washington, DC, ²American University, Washington, DC

Recent reports and pedagogical research has suggested that an open-ended research experience can help science undergraduates develop problem-solving skills, work within a team, communicate science, and develop their own scientific interests. Unfortunately, many institutions are unable to sustain access for multiple undergraduates in faculty-led research programs for many reasons – including lack of resources and significant teaching loads for faculty. An alternative to students doing individual research projects is the creation of course-based research experiences (i.e., CUREs), which are proven methods to provide students with significant research experiences leading to the development of the skills and attitudes described above. Here, we propose a third alternative, the creation of the undergraduate teaching assistants, or UTAs. American University is a mid-size liberal arts university with a growing research faculty and student body interested in medicine, basic research and public health. In the department of Biology, Master's Students serve as teaching assistants for the laboratory portion of the introductory and non-major courses. Two years ago, we added UTAs to laboratories that were particularly technique heavy or where students struggled with fundamental concepts. Currently, UTAs participate in five of the 12 laboratories each semester and in TA training prior to each lab. Since the beginning of the UTA program, we have surveyed the UTAs pre- and post-semester as well as asked students taking the course to comment on the value of their UTA. We have found that UTAs report increased interest in science, increased confidence in trouble-shooting and understanding of fundamental concepts in biology. Students enrolled in laboratories served by UTAs report an average of 6 contact points with their UTA per semester. We have also found that the ease with which UTAs are recruited and trained has provided a mechanism for the more frequent involvement of women and under-represented minorities in the biology labs. We propose that the UTA position is an efficient, effective strategy to 1) teach undergraduates the skills often learned in a traditional research lab, 2) augment the pedagogical quality of laboratories and 3) increase the participation of underrepresented populations, including transfer and first-generation college students, in the sciences.

P825**Board Number: B138****Mouse Color Genetics as an Undergraduate Laboratory Exercise.**L.P. Lyman-Henley¹, L.E. Bailey¹;¹Biological Environmental Sciences, Texas AM University-Commerce, Commerce, TX

Finding laboratory exercises that engage undergraduate students in introductory biology and zoology courses can be challenging, especially when attempting to incorporate hands-on training in basic

research that stimulates critical thinking. I have developed a basic genetics exercise which beginning undergraduate students can perform in a 6 to 8 week period that allows them to learn basic inheritance patterns, collect data to test a hypothesis, and write a complete research-style laboratory report, which students find both educational and engaging.

“Mousy Match-Maker” allows students working in groups to choose from available live lab mice demonstrating a variety of coat colors and types. Students learn about phenotype and genotype in describing their chosen mice and referring to published information about mouse color genetics to attempt to identify the alleles represented by their subjects. Students then form hypotheses regarding the alleles that should be exhibited by the offspring of their mouse pair, following at least 2 distinct genes. Mice have a 21 day gestation period, so young are generally born within a month of pairs being introduced, and students can determine the coat color of offspring in another 2 to 3 weeks. Students can then record the actual coats of their young mice, and compare that to their predictions using Punnett squares. Chi-square tests are easily utilized to compare observed to expected results, and students can use “surprise” results to revise their original hypotheses.

One full lab period is devoted to the project at start up and at end. In the intervening weeks other lab exercises are scheduled, with brief weekly “installments” where students work on specific aspects of the project and check up on the progress of their mice. All of this makes an excellent project to describe in a scientific paper format while illustrating basic inheritance patterns, and generates far more interest and effort than any other “pre-packaged” genetics laboratory exercises used.

P826

Board Number: B139

A new course and textbook on Physical Models of Living Systems, for science and engineering undergraduates.

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I'll describe an intermediate-level course on "Physical Models of Living Systems." The only prerequisite is first-year university physics and calculus. The course is a response to rapidly growing interest among undergraduates in a broad range of science and engineering majors.

Students acquire several research skills that are often not addressed in traditional courses:

- Basic modeling skills
- Probabilistic modeling skills
- Data analysis methods
- Computer programming using a general-purpose platform like MATLAB or Python
- Dynamical systems, particularly feedback control

These basic skills, which are relevant to nearly any field of science or engineering, are presented in the context of case studies from living systems, including:

- Virus dynamics
- Bacterial genetics and evolution of drug resistance
- Statistical inference
- Superresolution microscopy
- Synthetic biology
- Naturally evolved cellular circuits

The poster will show examples of student work from a mid-course "capstone" project involving modeling the Luria-Delbrück experiment via simulation-based likelihood maximization. The project is partly open-ended; examples will be shown of work by students who have never done computer programming prior to this course, as well as more sophisticated students who took the assignment farther. Outcomes include student reports of improved ability to gain research positions as undergraduates, and greater effectiveness in such positions, as well as students enrolling in more challenging later courses than they would otherwise have chosen.

P827

Board Number: B140

The FUTURE Program: Ensuring that underserved populations become the scientific and civic leaders of tomorrow.

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The Center for Science and the Common Good (CSCG) at Ursinus College provides a framework for all Ursinus science majors to think, speak, and write about the impact of science on the common good. One target group of the CSCG is the FUTURE Students (Fellowships in the Ursinus Transition to the Undergraduate Research Experience), members of underserved populations in science who are given research opportunities early and often to ensure that they can become scientific and civic leaders. We include underrepresented minorities (URMs), women, first-generation college students, and disabled students in this program. Students in the program enter a research experience during the summer prior to or directly after their first college year. During this time, they participate in a team-taught course titled "Science and Mathematics in Society" and are closely mentored by a faculty member and an upper class FUTURE Mentor. FUTURE students are encouraged to continue in research following the summer experience, to become FUTURE Mentors, and to participate fully in the CSCG. The CSCG was founded in 2012 through an HHMI grant and we have run two successful summer experiences to date. All FUTURE participants have continued in STEM fields. Through use of the national survey of undergraduate research experiences (SURE), we have shown that students who have participated in the FUTURE program show gains in all areas that are at or above the national average. Some positive impacts on students include: making them more resolute in their career choice, giving them confidence in their ability to communicate and make connections with faculty members, and increasing their awareness of the role of science in the common good.

P828**Board Number: B141****Isolating and analyzing bacteriophages with first year Biology students at Saint Joseph's University.**J.Y. Lee-Soety¹, C. King-Smith²;¹Biology, Saint Joseph's University, Philadelphia, PA, ²Department of Biology, Saint Joseph's University, Philadelphia, PA

Saint Joseph's University has been a member of the SEA-PHAGES (Science Education Alliance-Phage Hunters Advancing Genomics and Evolutionary Science, supported by the HHMI) consortium since 2009. We are in our fifth year of leading novice scientist, first year students majoring in Biology, Chemical-Biology, Math, and Environmental Sciences, in isolating, naming, sequencing, and genomic annotation of bacteriophages. From collecting soil to calling genes over the course of an academic year, the students are involved in authentic research using cutting-edge techniques. In the first semester, students isolate and purify novel phages from soil samples; they then characterize the phages by electron microscopy, DNA restriction digests, and pulsed-field gel electrophoresis. In the second semester, fully sequenced phage genomes are annotated using bioinformatics tools. We have now isolated 54 mycobacteriophages that infect *Mycobacterium smegmatis*, and 11 of those have been completely sequenced and analyzed. Five fully annotated sequences have been published on GenBank (Mycobacteriophages Daisy, BPBiabs31, Flux, Winky, and Oaker). This year, we have ventured to explore a new bacterial host – *Arthrobacter* sp. Both *Arthrobacter* sp and *M. smegmatis* are soil bacteria and from the same Actinomycetales order. So far, we have isolated 22 *Arthrobacter* phages and sequenced two. In addition to experiencing first hand research, students are exposed to primary journal articles and presenting their work orally and in writing; all of these activities building upon interests in the sciences and help with student retention in the STEM majors.

P829**Board Number: B142****Integrating authentic research in cell biology into an undergraduate liberal arts curriculum to enhance the pace of rare disease gene discovery for underserved populations and promote STEM retention.**R.N. Jinks¹, B.K. Davis¹, D.M. Roberts¹, E. Rice¹, K.W. Brigatti¹, E.G. Puffenberger², K.A. Strauss²;¹Franklin Marshall College, Lancaster, PA, ²Clinic for Special Children, Strasburg, PA

Next-generation sequencing accelerates disease gene discovery, especially for orphan diseases, though at present it outpaces functional studies needed to provide 'proof of causation.'

Working from a small, community-based, clinical laboratory, the Clinic for Special Children has identified more than 130 allelic variants associated with disability, disease, or untimely death among the Amish and Mennonite (Plain) populations of North America. In collaboration with the Clinic, we developed an

HHMI-funded program that integrates functional studies of novel disease gene alleles into our undergraduate curriculum. Roughly 150 students per year in our introductory cell biology and neuroscience courses clone human disease genes and study the functional impacts of gene variants through expression in mammalian cell culture. We use these authentic research experiences to teach key concepts in cell biology, genetics, and neuroscience. Students later build upon this experience in upper-level courses in cell biology, neuroscience, genetics, cancer biology, and immunology in which they engage in semester-long research projects in small teams. Teams conduct functional studies of disease gene variants or study novel cellular or animal models of disease. These experiences engender talented undergraduates to assume greater research responsibility through independent study and summer research projects that transition them into the role of co-PI for their project. Data published through this project (PLoS ONE 7:e28936) were recently used by another institution to diagnose a critically-ill, non-Plain newborn with lethal neonatal seizure-rigidity syndrome (Sci Transl Med 4:154ra135). This provides important proof-of-concept for integrating novel disease gene functional studies into a carefully structured undergraduate research curriculum. Students in our Public Health program are collaborating with the Clinic to develop handbooks to help parents care for children with special medical needs with the goal of producing one to two high-quality disease handbooks annually. Our program represents a model for engaging undergraduates in meaningful research at the front lines of biomedical science and public health in ways that directly impact the diagnosis and care of children with rare inherited disorders and promote STEM retention.

P830

Board Number: B143

Development of a novel, inquiry-based curriculum for the undergraduate cell biology laboratory.

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Research in undergraduate scientific education has shown that the most effective methodology in teaching laboratory-based science is to utilize an applied approach that emphasizes both inquiry and the use of the scientific method to solve “real world” problems. Student learning is most positively impacted by the design and execution of longer-term projects that more closely mirror the types of investigations that are carried out by actual research scientists. This is in contrast to a “cookbook-like” sampling of techniques, which are often taught in succession without a direct context for application to scientific research. Muscle differentiation is a complex process that provides a unique opportunity for undergraduate students to explore various aspects of cell biology in the laboratory. The work presented here describes the development of a novel inquiry-based undergraduate cell biology laboratory course focused on the examination of muscle cell differentiation. In this innovative curriculum, students were involved in the entire scientific process, from asking the experimental question to designing and performing the experiments, and then analyzing the results. They utilized a variety of complimentary techniques (including fluorescence microscopy, western blotting, and polymerase chain reaction) to

examine the morphological and genetic changes that occur during muscle cell differentiation in culture. Unlike typical labs which usually have an objective that is met in one or two class sessions, the students engaged in a longer-term project that spanned eight weeks during the semester. Students were assessed on the quality of their laboratory notebooks, including how thoroughly they documented each experiment and answered related discussion questions regarding the techniques and results. Ultimately, students compiled their work into an individually written research report, the format of which paralleled typical journal articles published in the field of cell biology. Overall, the design of this curriculum allowed students to explore fundamental cell biology topics while learning key experimental techniques. Furthermore, this was placed into the context of a conventional research experience to more effectively engage students in learning through direct inquiry and investigation.

Imaging Technologies, Single Molecule Imaging, and Super-Resolution 2

P831

Board Number: B145

MULTI-MICROBEAM SYSTEM FOR LOCALIZED INDUCTION OF DNA DAMAGE BY MULTIPLE TYPES OF RADIATION AND LIVE CELL IMAGING OF CELLULAR RESPONSES.

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KEY WORDS: DNA damage, live cell imaging

Cellular DNA damage responses (DDR) are important in cancer induction, diagnostics and treatment. Therefore, understanding of DDR pathways is crucial for improvement of existing and development of novel anti-cancer therapies. “Localized” DNA damage, induced in a small volume of the cell nucleus, is an important tool because signaling and repair responses triggered by the localized DNA damage can be studied in great detail using microscopical techniques not only in fixed cells, but also in living cells with fluorescently-tagged DNA repair-related factors. Induction of DNA damage using focused UV laser beams is a commonly applied technique, but there is a growing concern about the poor characterization of the DNA lesions produced by these sources. This is an important issue, since different repair processes are taking care of different types of DNA damage. In order to overcome this drawback, we constructed a novel type of instrument, coined the multi-microbeam (MMB). This instrument can be equipped with various irradiation sources to induce specific DNA damage. An ultra-soft X-ray source is used to induce DNA double-strand breaks (DSBs). The ultra-soft X-ray source is positioned under an

ultra-thin bottom culture dish containing adherent cells. A metal mesh filter with a dense array of micro apertures is positioned between the radiation source and the culture dish, enabling local irradiation of cells. A Leica SP5 confocal microscope, equipped with a water dipping objective, is positioned above the source, allowing real-time observation of cellular responses. A large part of the microscope and the irradiation source is enclosed in an incubator with CO₂ and temperature control. Using the MMB the accumulation kinetics of a number of DSB factors on sites of DNA damage can be analyzed. The system also allows Fluorescence recovery after photo-bleaching (FRAP) experiments to establish the residence time of proteins at the damaged DNA. A prototype of the MMB was already used to detect recruitment of heterochromatin protein 1 to DSBs. We expect that MMB irradiation will serve as a standard, given the well-characterized damage spectrum of the irradiation sources.

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P832

Board Number: B146

Live cell imaging of cytoplasmic mRNP complexes in response to HIV-1 infection.

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In mammalian cells, dynamic cytoplasmic messenger ribonucleoprotein (mRNP) complexes form in response to cellular stressors, including viral infection. These mRNPs include processing bodies (PBs) and stress granules (SGs), sites of RNA decay and suppression, respectively. Many RNA viruses have evolved mechanisms to evade or coopt PB and/or SG machineries, including the human immunodeficiency virus type 1 (HIV-1). To directly monitor the effects of HIV-1 infection on cytoplasmic mRNPs, we generated stable cell lines expressing visible versions of PB markers (APOBEC3G, Ago2, and Dcp1a) and SG markers (TIA1 and G3BP) and infected these cell lines with HIV-1 or the murine leukemia virus (MLV), both retroviruses, or the picornavirus rhinovirus A. Cells were monitored over the course of infection (over 72 hours) for changes to the quantity, morphology, and distribution of PBs and SGs over time. For HIV-1, this approach yielded single cell dynamics for HIV-mediated downregulation of the antiviral PB protein A3G by the HIV-1 protein Vif. We found this process to take more than 60 minutes per cell. In the absence of the HIV-1 Vif protein, infection led to remarkable relocalization of A3G from PBs and the cytoplasm to the plasma membrane. We observed A3G aggregating in large, 1-2µm diameter mRNP complexes at the cell surface containing the HIV-1 Gag capsid protein. Interestingly, we also noted that HIV infection can induce the transient formation of SGs and, unexpectedly, causes a marked condensation of TIA1 into bright nuclear foci at late times post-infection. Taken together, we have generated a tractable visual system for monitoring single cell RNP responses to viral infection, and confirm that HIV-1 infection can trigger dynamic remodeling of PBs and SGs of potential relevance to the viral life cycle.

P833**Board Number: B147****Super-constriction precedes GTP-hydrolysis dependent conformational changes in dynamin polymers during plasma membrane fission.**A.C. Sundborger¹, J.E. Hinshaw²;¹NIH, NIDDK, Bethesda, MD, ²NIH/NIDDK, Bethesda, MD

Dynamin is a mechanochemical enzyme that assembles into short scaffolds on necks of budding vesicles to mediate plasma membrane fission during clathrin-mediated endocytosis. Recent structural data has revealed key features of dynamin function during fission: 1) stalk-driven oligomerization into polymers with helical symmetry 2) dimerization of G domains that stabilizes the transitions-state of the GTP hydrolysis-reaction, a pre-requisite for productive fission, and 3) a GTP-dependent conformational change in the polymer referred to as the dynamin powerstroke. We show that dominant-negative dynamin mutant, K44A, is defective in stimulated GTP hydrolysis due to its inability to stabilize the GTP hydrolysis transition state. K44A polymers are trapped in a pre-fission state, capable of “super-constricting” necks of budding vesicles to the point of spontaneous fission. Super-constriction is achieved by K44A adopting a 2-start helical symmetry, which allows the polymer to constrict the underlying lipid bilayer to an inner luminal diameter of only 3.7 nm. We further show that super-constricted K44A is trapped in a ground state-configuration, which indicates that GTP binding is sufficient to promote an initial conformational change in the polymer, prior to GTP hydrolysis. In the super-constricted state, half of the PH domains are tilted out of the membrane compared to the non-constricted dynamin polymer. This suggests that upon super-constriction, the PH domain re-arranges its orientation in the outer leaflet of the bilayer, which may contribute to destabilization of the lipid, and act to promote fission. To understand how GTP hydrolysis ultimately promotes fission we performed time resolved studies of WT dynamin using cryo-EM. We show that like K44A, WT dynamin assembles into a 2-start helix in the presence of GTP that super-constricts the underlying lipid to the point of spontaneous fission. We further show that super-constriction is followed by a series of distinct conformational changes that ultimately lead to twisting, super-coiling, disassembly and fragmentation of protein-decorated lipid tubes. Therefore, we suggest that a fundamental function of dynamin is to assemble into a 2-start helix around necks of budding vesicles. This allows for super-constriction of the neck, optimizes G domain organization in the polymer, and evokes stimulated GTP hydrolysis that triggers a series of conformational changes; the BSE powerstroke, PH domain re-arrangement, loosening of the polymer and ultimately fission.

P834**Board Number: B148****Measuring mesoscale dynamics in chromosome organization with single-molecule tracking correlation spectroscopy.**C. Limouse¹, C. Fuller², J. Bell², A.F. Straight³, H. Mabuchi⁴;¹Stanford University, Stanford, CA, ²Biochemistry, Stanford University, Stanford, CA, ³Biochemistry, Stanford School of Medicine, Stanford, CA, ⁴Applied Physics, Stanford University, Stanford, CA

Numerous cellular processes involve conformational changes occurring at the scale of tens to hundreds of nanometers. The most compelling example is perhaps genomic organization where transient contacts between remote loci separated by hundreds or thousands of bp are ubiquitous and essential for transcription regulation. These processes are difficult to probe both *in vivo* and *in vitro* because they are sub-diffraction limit yet too large to be easily accessible by precise single-molecule methods such as FRET. I discuss the application of single-molecule fluorescence correlation techniques, as well as single-molecule tracking as a way to resolve conformational dynamics on these length scales. I present a proof-of-principle experiment where we measured the end-end dynamics of short DNA fragments freely diffusing in solution. Finally I discuss future applications to the detection of DNA looping induced by CTCF and other DNA binding proteins.

P835**Board Number: B149****Determination of sample preparation parameters critical for optimal Structured Illumination Microscopy (SIM).**C. Arvanitis^{1,2}, N.J. Dolman³, J.Z. Rappoport^{2,4};¹Pathology, Northwestern University, Chicago, IL, ²Center for Advanced Microscopy, Northwestern University, Chicago, IL, ³Molecular Probes Labeling and Detection Technologies, Thermo Fisher Scientific, Eugene, OR, ⁴Department of Cell and Molecular Biology, Northwestern University, Feinberg School of Medicine, Chicago, IL

Super-resolution microscopy is a term used to describe a variety of techniques that bypass the diffraction limited resolution of a light microscope (~250nm). Structured Illumination Microscopy (SIM) is a super-resolution microscopy technique by which one can obtain roughly double the resolution of standard light microscopy. SIM accomplishes this by using a grid to generate a Moiré pattern on the sample. The grid is moved and rotated resulting in a total of 9-15 images, which are then computationally reconstructed to generate a final image with a resolution of roughly 125 nm. The quality, and resolution of this final image is highly dependent on sample preparation. Photobleaching, signal from out of the focal plane, spherical aberration (introduced by cell structures such as nuclei or poor quality mounting media) and low signal to noise can result in images which are filled with artifacts or not resolved. To date there are no studies which determine the optimal mounting media for SIM

microscopy, in particular across different samples and SIM imaging platforms. Here we compare results from two different SIM microscopes (Nikon N-SIM and GE OMX) using both sub resolution beads and fluorescently labeled cells mounted in media from a variety of manufactures, as well as a homemade buffered glycerol solution containing p-Phenylenediamine (PPD). For bead preparations, we measured maximal resolution using full width half max (FWHM) measurements in four channels (405, 488, 561 and 640 excitation). To model more relevant biological samples, cells were grown on coverslips loaded with 20nm red FluoSpheres and labeled with Alexa Fluor 488 phalloidin. FWHM measurements were taken on both the FluoSpheres as well as actin filament diameter to compare resolution within cells. Results from each of the different sample preparations are presented.

P836

Board Number: B150

Combination of Stimulated Depletion Emission (STED) microscopy with Electron Microscopy (EM) to image the uptake and trafficking of A β oligomers into murine Microglia.

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The diffraction limit (D_{xy}) is the minimum distance required between two objects to resolve them individually and is defined by the wavelength of the excitation light (λ) and the numerical aperture of the lens (NA) ($D_{xy} = \lambda / 2NA$). Microscopy has entered a new era with technological advances in super-resolution imaging which allows for the achievement of a resolution beyond the lateral diffraction limit of 250 nm. One of these approaches is STED microscopy based on Stefan Hell's concept, in which the resolution further depends on the ratio of the intensity of the excitation laser versus the intensity of the depletion laser (I_{sat}): $D(xy) = \lambda / 2NA (1 + \sqrt{1 + I/I_{sat}})$. The Leica SP8 STED 3X system can image samples labeled with up to 3 different fluorescent tags down to maximal xy and z resolutions of 45 nm and 130 nm, respectively. This increased resolution dramatically improves discrimination, description, and quantification of sub-cellular compartments. Cell trafficking is a crucial question relevant to numerous biological fields of investigation. In this study, we show that gated STED imaging allows to routinely increase the lateral resolution by a factor of 2 compared to confocal imaging and that further deconvolution using Huygens software improves the resolution an additional 3 fold. We have applied this technology to study the internalization of A β oligomers by murine microglia. A β oligomers are considered as one of the hallmarks of Alzheimer's disease and have been suggested to contribute to disease pathophysiology. In addition, uptake of A β oligomers by microglia is thought to be involved in oligomer clearance from the brain and may play a critical role in disease progression. Here, we studied A β oligomers uptake and intracellular trafficking into microglia using 3 dimensional STED imaging. We performed double immunofluorescence for A β oligomers in combination with endocytic markers such as clathrin, caveolin, LAMP, EEA1 as well as the autophagocytic marker LC3 in microglia. The specificity of the markers was further confirmed by immuno-electron microscopy. Correlating STED images with

electron microscopy images (CLEM) provides additional cellular morphological information and context. Altogether, these results demonstrate the feasibility of combining STED and CLEM and allow the identification of the subcellular trafficking of A β oligomers in microglia at a resolution never achieved previously.

P837

Board Number: B151

iSERS Microscopy: Multiplexed Protein Localization for Tissue-based Cancer Diagnostics by Surface-Enhanced Raman Scattering.

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Optical localization of proteins by microscopy is often performed by usage of fluorescent labels. Proteins can be labeled with fluorescent dyes directly or indirectly by antigen-recognizing fluorophore-labeled antibodies (immuno-fluorescence). In cases where multiple targets have to be colocalized, the number of distinct fluorophores is limited. This is due to the huge overlap of the broad spectral emission profiles of available fluorescent dyes. Parallel detection of more than four fluorescence colors is therefore very challenging. In contrast, Raman emission profiles are characterized by narrow line widths and can even be used parallel to established fluorescence detection. In combination with Raman-active noble metal nanoparticles, the surface-enhanced Raman scattering effect (SERS) can be exploited which results in an increase of the vibrational Raman intensity by several orders of magnitude.^[1] This offers multiplexed and quantitative detection of numerous cellular entities which can be performed with up to dozens of different Raman-"colors" using a single laser excitation wavelength. The properties of the colloidal SERS probes, like signal brightness, stability and robustness, are important for this task. Multiple SERS-labeled antibodies can be used for the parallel detection of numerous antigen targets on tissue biopsies, enabling fast multi-testing of different cancer antigens by immuno-SERS microscopy (iSERS).^[2] Two-(Raman)-color iSERS experiments localizing the tumor suppressor p63 and prostate-specific antigen (PSA) on prostate tissue samples show the possibility of fast cancer antigen detection on large biopsy areas. The differentiation of histological subclasses, like p63(+) basal epithelium and p63(-) secretory epithelium, is visible in high resolution iSERS-images.^[3] Results of multiplex-iSERS imaging experiments on tissue samples, targeting different protein antigens, are presented.

References [1] Schlücker, S. (2014). Surface-Enhanced Raman Spectroscopy: Concepts and Chemical Applications. *Angew Chem Int Edit* 53, 4756-4795. [2] Schlücker, S. (2009). SERS Microscopy: Nanoparticle Probes and Biomedical Applications. *Chemphyschem* 10, 1344-1354. [3] Salehi, M., Schneider, L., Ströbel, P., Marx, A., Packeisen, J., and Schlücker, S. (2014). Two-color SERS microscopy for protein co-localization in prostate tissue with primary antibody-protein A/G-gold nanocluster conjugates. *Nanoscale* 6, 2361-2367.

P838**Board Number: B152****Novel mouse xenograft model for noninvasive in vivo imaging of human tumor cell and tissue in the auricle.**S. Kita¹, H. Higuchi¹;¹Physics, The University of Tokyo, Tokyo, Japan

We developed methods of preparing xenograft model and imaging GFP-expressing cells to observe noninvasively cells in mouse auricles. In the previous studies, we imaged invasively the tumor exposed by dissection of skin and epithelium. By the dissection, blood flow was blocked, resulting in lack of supply of oxygen and nutrient. We, however, concern if homeostasis of their cells is maintained to elucidate biological phenomenon in imaging under ischemic condition. Therefore, we developed new noninvasive imaging methods. We selected the ear auricle of mouse for observation of tumor cells because of very thin (about 150-200 μ m) and limited hypodermal tissue. We have developed a novel xenograft model which has tumor in auricle. SCID mice of 8-12 weeks old were used in our experiments. We injected five kinds of human cancer cell lines into the ear auricle of SCID mice; breast cancer cell lines named KPL4-EB1-GFP and MDA-MB-231, MDA-MB-231-EB1-GFP and MDA-MB-231-GFP-tub, and glioma cells line U87MG. Tumor was successfully formed 100% of injected mice at incitation of $> 4.6 \times 10^6$ cells of KPL4-EB1-GFP, which is known to form tumor tissue easily on subcutaneous. On the other hand, MDA-MB-231 cell line had been difficult to form tumor at subcutaneous of mouse back. Surprisingly, we successfully develop the xenograft about 100 % of injected mice by inoculation of $> 4.0 \times 10^6$ cells of MDA-MB-231, MDA-MB-231-EB1-GFP and MDA-MB-231-GFP-tub in the ear auricle of mice. Tumor formation rate of U87MG which is known as cancer having high malignancy was 100% at injected more than 0.8×10^6 cells. The tumor and cells in auricle were noninvasively imaged by spinning disk confocal (CSU) system equipped with automatic positioning stage, piezo actuator for objective and an EMCCD camera. We imaged GFP fluorescence in the MDA-MB-321-GFP-tub cells in tumor of ear auricle without injuring mice. The individual two cells in tumor were distinguished faintly with bright background of tumor fluorescence. We also took a montage view of tumor cover wide area (3x2 mm). The shape of a tumor appeared faintly at the depth $> 40 \mu$ m, suggesting the shape is background of a tumor located deeper. There are several bright spots in the diameter of about 20 μ m in the enlarged image, indicating those are single cells. We could successfully perform real time observation of GFP fluorescence in the breast cancer cells in noninvasive condition by a CSU system.

P839**Board Number: B153****Multiplex imaging of DNA damage in cell suspensions.**A. Didenko^{1,2};¹Vivid Technologies Inc, Houston, TX, ²Tufts University, Medford, MA

We developed a new approach to study cellular reactions in cell suspensions via multiplex imaging of DNA alterations. The approach detects conditions of individual cells by visualizing DNA-centered biomarkers. For this, the cells are initially immobilized on a set of selective capture membranes and are then analyzed *in situ* for the presence of specific types of DNA breaks, which indicate apoptosis, phagocytosis or non-lethal DNA damage. The methodology is fast and cost-effective. It is advantageous in research using complex cellular suspensions composed of cell types with dissimilar responses to stressors. The method is a new multiplex imaging tool useful in molecular and cell biology.

P840**Board Number: B154****Optogenetic Control of Fibroblast Growth Factor Receptor Signals.**M. Lee¹, N. Kim², J. Kim¹, C. Kim¹, K. Chang², W. Heo^{1,2};¹KAIST, Daejeon, Korea, ²IBS, Daejeon, Korea

Fibroblast growth factor receptors (FGFRs) regulate diverse cellular behaviors such as proliferation, growth, differentiation, migration, survival, and apoptosis. Here, we present a synthetic approach to modulate FGFR signaling by light in a highly spatiotemporal manner. We engineered an optically controlled FGFR (optoFGFR1) by exploiting cryptochrome 2, which homointeracts upon blue light irradiation. OptoFGFR1 can regulate the signaling pathways of FGFR by light in a rapid and reversible manner with subcellular resolution. At the single cell level, localized effect of optoFGFR1 induced changes in the cytoskeleton. Utilizing the precision control of optoFGFR1, we can induce cell polarization and directed cell migration. With the same scheme of optoFGFR1, we developed optoFGFR2, optoFGFR3 and optoFGFR4 (optoFGFRs) for investigating FGFR subtypes mediated signaling pathways. The genetically modified light-inducible FGFRs, optoFGFRs, shed us a potential on the study of FGF signaling in various biological systems.

P841**Board Number: B155****Live-cell imaging of RNAs fused to Spinach using fluorescent microscopy in *Saccharomyces cerevisiae*.**J.A. Zinskie¹, M. Bruist¹, C.J. Janetopoulos², K.A. Myers³;¹Chemistry Biochemistry, University of the Sciences, Philadelphia, PA, ²Biological Sciences, Univ of the Sciences, Philadelphia, PA, ³Department of Biological Sciences, Misher College of Arts and Sciences, University of the Sciences, Philadelphia, PA

A wide array of options exists for live-cell imaging of RNA, however each option has its own drawback. A recent development in the field of RNA imaging is the development of a small -RNA aptamer, Spinach, which interacts with a fluorophore to produce fluorescence. To test this system in *Saccharomyces cerevisiae*, yeast, a small-nucleolar gene, SNR30, was fused to the Spinach aptamer. The gene fusion was expressed in and imaged using fluorescent microscopy. Spinach did not affect RNA localization and yielded a high quantum yield of fluorescence. Additional small-RNAs have been tagged with the Spinach aptamer, expressed in yeast and visualized using fluorescent microscopy.

The ability to visualize subcellular organelles in yeast cells has required fixation due to Brownian motion. Immobilization by embedding live yeast on the surface of 2% agarose has been used, but gives poorer resolution than fixation. Agarose also reduces the signal intensity, yielding a less than optimal image. To combat this reduction in image quality, a new microcompressor has been used for live-cell fixation; considerable subcellular detail can be captured from such mechanically fixed cells.

P842**Board Number: B156****Signaling Events and Cellular Processes Modulated by Ultra-small Integrin-targeted Silica Nanoparticles in a Concentration-dependent Manner.**M. Benezra¹, M. Pauliah¹, M.H. Overholtzer², U. Wiesner³, M.S. Bradbury^{1,4};¹Department of Radiology, Memorial Sloan-Kettering Cancer Center, New York, NY, ²Cell Biology Program, Memorial Sloan Kettering Cancer Center, New York, NY, ³Department of Materials Science and Engineering, Cornell University, Ithaca, NY, ⁴Molecular Pharmacology and Chemistry Program, Sloan Kettering Institute, New York, NY

Ultra small multifunctional silica nanoparticles may provide a highly versatile disease-directed platform for delivery of therapeutic drugs. Of critical importance to the clinical advancement of such platforms is the need to completely characterize novel particle probes at the cellular and molecular levels, and elucidate how particle surface chemistry potentially modulates key biological properties of these probes. In this study, we characterized these new fluorescent particles, termed Cornell dots (or C dots surface-functionalized with polyethylene glycol (PEG) chains for conjugation of cyclic arginine-glycine-aspartic acid-tyrosine (cRGDY) peptide ligands). We have previously shown high binding affinity, and

enhancement of potency, and multivalency with this ultra small, inorganic nanoparticle platform. In this work we investigate mechanism of action, subcellular internalization, and expression levels of key signaling pathway intermediates in the alpha-v-beta-3 ($\alpha v\beta 3$) integrin-expressing human melanoma (M21) and umbilical vein endothelial (HUVEC) cells as a baseline for future therapeutic work. Concentration- and time-dependent binding was observed by flow cytometry and gamma counting using cRGDY-PEG-C dots in M21 and HUVEC cells. PEG-C dots served as the control. Saturating conditions were achieved at room temperature after incubating M21 and HUVEC cells with 100 nM of the dots for 4 hours. Binding specificity of the targeted platform was also demonstrated relative to the control probe by integrin receptor blocking using the native cRGD peptide as a competitor. Importantly, we observed activation of multiple signaling pathways in M21 cells upon targeted particle binding. Phosphorylation of focal adhesion kinase (FAK), led phosphorylation of Src, accompanied by additional phosphorylation of FAK. FAK phosphorylation induced activation of downstream signaling pathways. Addition of a FAK inhibitor to M21 cells incubated with cRGDY-PEG-C dots blocked phosphorylation of FAK and multiple downstream effectors. Additionally, enhanced in vitro migration rates were seen upon addition of cRGDY-PEG-C dots to both HUVEC and M21 cells, and a higher percentage of M21 cells were observed in S phase relative to that measured under control conditions. Furthermore, observed alterations in the adhesion and spreading properties of M21 cells, manifested, as cellular morphologic changes in a competitive binding assay, may, in part, be the result of pathway modulation upon particle exposure. Taken together, these data suggest that multiple FAK-mediated, integrin-stimulated signaling pathways are activated in M21 and HUVEC cells after exposure to particles. These changes, in turn, modulate key biological processes- cell migration, adhesion/spreading, and proliferative activity- known to be critical to angiogenesis, metastatic invasion, and wound healing.

P843

Board Number: B157

Bioluminescence resonance energy transfer (BRET) image analysis of Ras-Raf interaction in live cells using NanoLuc luciferase and Venus yellow fluorescent protein by bioluminescence microscopy.

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NanoLuc (Promega) is a new luciferase originated from deep sea shrimp and emits blue bioluminescence utilizing imidazopyrazinone substrate. Since NanoLuc is approximately 150 fold brighter than firefly luciferase, a bioluminescent image of cells expressing NanoLuc can be captured within a sub-second exposure time by a bioluminescence microscope, LV200 (Olympus) equipped with an electron multiplying (EM)-CCD camera (ImagEM, Hamamatsu Photonics). We used NanoLuc and a yellow-emitting mutant of *Aequorea* GFP (Venus) as the donor and acceptor of bioluminescence resonance energy transfer (BRET), respectively, to image activation of Ras, a small G protein that transduces signaling from receptor tyrosine kinase to Raf (MAP kinase kinase kinase) at the inner part of cell membrane. H-Ras and Ras-binding domain (RBD) of Raf-1 were fused to Venus and NanoLuc,

respectively. Venus-Ras and RBD-NanoLuc were co-expressed in HeLa cells. After substrate administration, bioluminescent images through the donor (435-495 nm) and BRET (530 nm long pass) channels were captured alternately using LV200 equipped with ImagEM. This experimental system allowed us to image Ras-Raf interaction with a temporal resolution of 10 seconds. Immediately after stimulation with EGF (epidermal growth factor), an increase in BRET signal occurred on the plasma membrane (PM). BRET increases due to Ras-Raf association were not only the PM but also intracellular vesicle. We clearly identified another increase in BRET signal appearing inside the cytoplasm at approximately 10 min after EGF stimulation. The latter activation of Ras involved endomembranous structures, such as Golgi apparatus and recycling endosome. To map the Ras activation locations more precisely, BRET-imaged samples are being subjected to immunocytochemical experiments that use organelle-specific antibodies. In this way, we demonstrate that BRET imaging using NanoLuc with the help of LV200, and ImagEM is a powerful means for studying spatiotemporal pattern of cellular events in live cells.

P844

Board Number: B158

Comparative phototoxicity of 488nm and 546nm light on the cell cycle progression of untransformed human cells.

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We systematically characterized the relative phototoxicity of 488nm and 546nm light on cell cycle progression of untransformed human (RPE1) cells. Unlabelled cells were irradiated by a 100 W high pressure mercury lamp on a Leica DMRE epifluorescence microscope through Leica L5 or Red-GFP band-pass filters and a 20X, 0.4 NA lens. Light intensities at the specimen plane were 13.6mW/cm² for 488nm and 20.6mW/cm² for 546nm. Mitotic shake off cells were plated on coverslips and 2 hours later (early G1) were given single dose irradiations ranging from 0.5 – 2.5 minutes. Irradiated fields were continuously followed for 48 hours (~2 normal cell cycles) to determine how many cells progressed to the next mitosis. Reduction in cell cycle progression was our measure of phototoxicity. All control cells entered mitosis under these conditions. For 488nm light we observed a dose dependent reduction on the percentage of cells that progressed to mitosis with only 16.4% dividing after 2.5 minute irradiations, as well as a dose-dependent delay in the time cells do reach mitosis. In contrast, equivalent irradiations of 546nm light (normalized for photons/second/cm²) resulted in 83% of cells progressing to mitosis. Preliminary results suggest that the introduction of a long-pass filter into the illuminating path to diminish possible leakage of <430nm wavelengths through the excitation filters produces no increase in the fraction of cells progressing to mitosis for any light dose of either wavelength. We also characterized the effect of spreading the total irradiation durations out into a series of 10 second pulses as well as conducting single longer, but lower intensity, exposures. Neither regime made a significant difference in the percentage of cells that progress into mitosis relative to the single acute dose regimes. Lastly, cells

expressing GFP-centrin 1 showed increased sensitivity to single 488nm exposures with 10% less cells entering mitosis at each exposure tested. In contrast, cells expressing mCherry-centrin 1 did not exhibit a significant change after single 546nm doses compared to WT cells.

P845

Board Number: B159

Orientation-Independent Differential Interference Contrast (OI-DIC) Microscopy can detect Mouse Sperm Acrosome Reaction.

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The quality of sperm has an effect not only on the IVF and ICSI outcomes but also on the incidence of abnormalities in their protoplasmic compartment and in the predisposition to genetic and epigenomic errors, which increases with oxidative stress. Before IVF and ICSI sperm quality must be estimated to assure normal embryo development. Unfortunately, most of the techniques used to investigate sperm cells are highly invasive and cannot be adopted to select the fertilizing spermatozoon.

We recently developed a new imaging mode called Orientation Independent Differential Interference Contrast Microscopy (OI-DIC). New system is implemented on research grade upright and inverted Olympus microscopes BX61 and IX81. The OI-DIC system can switch DIC shear directions by 90° and change bias in about 50 msec. Within 1 sec the OI-DIC captures 6 raw DIC images with two orthogonal shear directions and three different biases. Then the obtained raw images are processed to generate the high resolution quantitative optical path length and gradient images in very thin optical section. In this study, we demonstrated that using OI-DIC microscope, the acrosomal status of mouse spermatozoa can be distinguished, and acrosome reacted spermatozoa can be selected for IVF and ICSI. Sperm were capacitated in BWW medium supplemented with (5mg/ml BSA and 20 uM bicarbonate) and then; acrosome reaction was induced with either Ca²⁺-ionophore, A23187 (5 uM) or progesterone (10 uM). The presence of acrosome (acrosome intact) in spermatozoa was visualized with OI-DIC system. Acrosome integrity was evaluated using Fluorescein conjugated *Pisum sativum* Agglutinin (PSA-FITC) with supravitality stain, Propidium Iodide (PI) by fluorescent illumination using 520–540 nm and 620 nm filters. Acrosome integrity assay, A23187 Ca²⁺-ionophore induced acrosome reaction was evaluated with PSA-FITC/PI double staining showed that reacted acrosome were present in (90.98 ± 0.7003, n=5) of viable spermatozoa. Using OI-DIC system, we observed that (91.50 ± 0.5000, n=5) of viable spermatozoa with reacted acrosome. There was a strong correlation (r= 0.8639) between these two methods. This study suggests that non-invasive imaging of intact and reacted acrosomes by OI-DIC microscopy technique can be used as criterion for healthy sperm selection. The use of the proposed instrumentation for ICSI could represent not only a diagnostic tool but also an accurate and novel method for sperm selection. This study could provide not only a strategy guiding the criteria for sperm selection but also a tool to better understand about sperm physiology, especially nuclear integrity. The evaluation of sperm head using OI-DIC and birefringence in sperm cells could expand the limitations of

current sperm selection criteria for ART procedures by contributing additional information on sperm acrosomal status after sperm capacitation and potential in the routine evaluation of the male factor, as well as modifying the technique of sperm preparation.

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P846

Board Number: B160

Rapid 3D Tissue Synthesis by Chemically Programmed Assembly.

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Methods to construct three-dimensional tissues of defined structure, from multiple cell types, with single-cell spatial resolution, and within physiologically relevant extracellular matrices (ECM) would greatly advance the study of developmental biology, cancer, and regenerative medicine. Here, we combine microscale direct writing of oligonucleotides with DNA-programmed assembly to reconstruct a tissue from the bottom-up and across centimeter distances in a manner that is amenable to long-term culture and imaging. The process begins by chemically remodeling the adhesive properties of individual cells with degradable oligonucleotide glue, allowing them to rapidly and specifically adhere to objects coated with complementary DNA sequences. Living cells are then rapidly assembled, layer-by-layer, onto a DNA-patterned substrate. Treatment with DNase releases fully assembled tissues directly into ECM gels for three-dimensional (3D) culture and observation. We demonstrate that this method enables the synthesis of diverse tissues with precise size, shape, composition and spatial arrangement within a slab of ECM. Finally, we demonstrate the use of the method to quantitatively analyze competitive and cooperative interactions between malignant and non-malignant mammary epithelial cells in the same tissue, as well as to assemble a precisely structured multicomponent tissue incorporating combinations of primary and transformed human endothelial, epithelial, and fibroblast cells into a single integrated 3D tissue.

P847

Board Number: B161

Dynamic micropatterning of cells on nanostructured surfaces using a cell-friendly photoresist.

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Nanoscale topographical features of the extracellular matrix (ECM) influence the behavior of cells including cell differentiation, migration, and proliferation. While how nanoscale topographical structures

affect various cellular behaviors has been active area of research, dynamics of cells on nanostructured surfaces has not been systematically examined. Here, we developed a new micropatterning method to control cellular dynamics on nanostructured surfaces using a cell-friendly photoresist poly(2,2-dimethoxy nitro-benzyl methacrylate-r-methyl methacrylate -r-poly(ethylene glycol) methacrylate) (PDMP), and studied how nanotopography influences spreading dynamics of cells. Surfaces containing nanoscale ridge/groove structures were fabricated by replicating a mold using a UV-curable resin polyurethane acrylate (PUA). The PUA substrates were coated with gelatin and subsequently spincoated with PDMP. Then, Microscope projection photolithography (MPP) was performed by illuminating light through a photomask inserted in a field diaphragm of the microscope. UV-exposed areas of PDMP thin films were selectively removed and nanostructured surfaces coated with gelatin were exposed. Cells seeded onto the patterned surfaces were selectively attached onto the UV-exposed area, resulting in single cell array formation. Finally, PDMP thin films surrounding cells were removed by briefly illuminating UV without photomask to trigger spreading of cells. And spreading dynamics of HeLa cells on nanostructured surfaces were monitored by interference reflection microscopy (IRM). In addition to the simple groove/ridge structures, spreading dynamics of complex nanostructured surfaces were quantitatively analyzed. This method will be useful to understand cellular behaviors under complex microenvironments.

P848

Board Number: B162

NovaMatrix-3D: an alginate-based 3D cell culture system.

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NovaMatrix[®]-3D is an alginate-based cell culture system comprising an alginate foam matrix and an alginate immobilizing solution. Cells are first suspended in a solution of sodium alginate that is then applied to the foam. As the foam absorbs the cell suspension, gelation occurs in situ as calcium ions are donated from the foam which effectively cross-links the added alginate. A hydrogel forms in the pores of the foam entrapping the cells throughout the foam. Cell immobilization occurs at room temperature and physiological pH. Cell localization and multi-cellular structures formed within the transparent gel can be visualized using confocal microscopy after vital staining of cells within the gel. A unique aspect of using an alginate-based foam matrix for 3D cell growth is that cells and intact multi-cellular structures can be retrieved for further analysis by adding calcium chelating agents such as citrate that dissolves the gel.

Results: We have cultured 22 different cell lines and primary cells in NovaMatrix[®]-3D. All cell lines tested formed multi-cellular structures. Cells do not have receptors that recognize alginate and for some cell types this may promote spheroid formation as the gel will be inert. Other cells require cell-matrix interaction to proliferate. This is achieved by using a peptide such as RGD (Arg-Gly-Asp) coupled to alginate, thereby facilitating a signaling cascade via integrin-RGD interactions. For some cell lines, the addition of RGD-alginate induced dramatic changes in cell proliferation and formation of multi-cellular

structures. In a separate set up experiment, the effect of x-ray radiation on cells cultured under different oxygen concentrations was investigated. The growth of spheroids inside the 3D matrix was determined by evaluating changes in spheroid volume. Spheroids cultured under 20% oxygen showed a significant reduction in volume increase after irradiation with 15 Gy, compared to the spheroids cultured under 5% oxygen. This study indicates that hypoxia increases the resistance of NHIK 3025 (human cervical carcinoma) spheroids against x-ray radiation and demonstrates the utility of the matrix in dose-response experiments.

Conclusions: NovaMatrix[®]-3D mimics a 3D model with the potential to approximate cell proliferation and architecture within tissues or tumors. Cells are uniformly entrapped into the foam matrix by a gentle and fast technique, and visualization can easily be performed. Cells form multi-cellular structures within the matrix and intact structures may be isolated under physiologic conditions. The immobilized cells can be treated with drugs or other agents since the alginate matrix is permeable for small and medium sized molecules, making NovaMatrix[®]-3D a versatile 3D cell culture system.

P849

Board Number: B163

PDMS-based 3D microfluidic in vitro culture System for cornea disease research model.

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TGFBI (transforming growth factor- β -induced gene)-linked corneal dystrophy is an autosomal dominant disorder caused by a point mutation in TGFBI gene on chromosome 5q31. Age-dependent progressive accumulation of mutant-TGFBI protein in the corneal stroma is a hallmark of TGFBI-linked corneal dystrophy and interferes with corneal transparency. A prevalence of granular corneal dystrophy type 2, major type of TGFBI-linked corneal dystrophy, has been estimated 8.25 per 10,000 persons in South Korea. Ideally the effect of pathologically determined this disease should be examined in the whole cornea of a living organism. However, no animal model of TGFBI-linked corneal dystrophy replicates the accumulation of mutant-TGFBI protein in cornea as the complex pathophysiological changes seen in patients. This is a severe limitation in the pathological and therapeutic implication studies of TGFBI-linked corneal dystrophy. Additionally, development and analysis of animal models can also be time-consuming and costly. Miniaturization technology, in particular microfluidics, has shown promise in overcoming these limitations and disadvantages. We report simple microfluidics 3-dimensional (3D) compartmentalized culture systems which have employed mixed culture of Keratocyte cells and epithelial cells in 3D gels (e.g., collagen I). This microfluidics system enables information-rich in vitro assays.

Microfluidics devices were fabricated using soft lithography. Polydimethylsiloxane (PDMS, Sylgard 184 Silicon Elastomer Kit, Dow Corning) was molded over the SU-8 master and sandwiched between

transparency film and weights to allow access to the ports. For compartmentalization, a “trident” shaped channel was designed. Human cornea fibroblast cells and epithelial cells cultured in DMEM supplemented with 10% FBS and penicillin/streptomycin. All cultures were maintained at 37°C in a humidified atmosphere containing 5% CO₂. For collagen sample preparation, the cells were trypsinized and counted (concentration 1×10⁶ cells/ml). Collagen was prepared at concentration of 1.3 mg/ml initially by an acidic collagen solution. To neutralize collagen using HEPES buffer, the buffer was mixed with same amount of acidic collagen solution (1:1 ration).

The cornea fibroblast cells (Keratocyte) and epithelial cell was cultured in microfluidic system. And also collagen was stained by immunocytochemistry. We presented a simple compartmentalized 3D culture model that supports the Keratocyte and Epithelial cell *in vitro*. In conclusion, our PDMS-based 3D microfluidics cell culture system may help overcome barriers to a limitation in a scientific method to investigate pathogenesis and treatments of TGFBI-linked corneal dystrophy

P850

Board Number: B164

Probing cancer cell migration on topographically reconstituted ECM nanofibers.

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Intravital imaging has emerged as the dominant imaging modality for the visualization of tumor cell migration *in vivo*. Intravital imaging has identified the phenotypes of cell behavior *in vivo* at single cell resolution. However, the complexity of the tumor microenvironment makes mechanistic analysis of its contributions to cell phenotype uncertain. Previously, we utilized micro-patterned extracellular matrix (ECM) substrates to study cancer cell migration properties in 1D, but due to the flat nature of ECM substrate on the glass coverslip, we could not faithfully capture the 3D topography of ECM substrate which cells encounter *in vivo*. Here, we developed a more realistic *in vitro* system using reconstituted ECM with cylindrical substrate topography as found in the tumor microenvironment *in vivo* and demonstrate that it supports the phenotypes of cell migration seen *in vivo*. First, we investigated the *in vivo* tumor ECM architecture in two mouse models: PyMT- and breast carcinoma cell-derived mammary tumors, using second harmonic intravital imaging. Tumor cells were found to move on fibronectin associated collagen I fibers. We found a wide distribution of collagen I diameters with the major peak falling in the range, 1.5-2 μm . Based on these *in vivo* observations, we engineered cylindrical poly(lactic-co-glycolic acid) (PLGA) nanofibers matching *in vivo* dimensions and coated them with fluorescently-labeled ECM components (collagen I, fibronectin). Remarkably, all of the emergent tumor cell behaviors seen *in vivo* were recapitulated on the 1.5-2 micron fibers suggesting that the fiber topography is a key contributor to cell behavior *in vivo*. Tumor cells in the presence of macrophages formed linear assemblies of alternating tumor cells and macrophages, resembling streams found *in vivo*. Average cell speed on these fibers reached 1 $\mu\text{m}/\text{min}$, with maximum speed reaching 4 $\mu\text{m}/\text{min}$, consistent with

previous observations made for tumor cell speed *in vivo*; and two times faster than speeds seen on 2D surfaces. Surprisingly, we discovered dynamic nuclear deformations during carcinoma cell migration on nanofibers, similar to nuclear deformation observed in cells squeezing through a narrow opening *in vivo*, suggesting that carcinoma cell nucleus is inherently plastic and ECM space constraint is not required for nuclear deformation.

P851

Board Number: B165

Osteogenic environment provided by hybrid scaffold comprised of stickyapatite and collagenous membrane.

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For covering large bone defect and controlling the morphology of regenerated tissues, scaffold is essential in tissue engineering. For bone tissue engineering, osteo-conductivity is essential characteristic of bone scaffold to help bone regeneration, and inorganic ceramic materials are main player in exhibiting osteo-conductivity of scaffold. So, inorganic ceramic materials such as hydroxyapatite are essential composition for bone scaffold. However, ceramic biomaterials itself show demerit of brittleness. In biological bone, polymeric structure of collagen counterbalance brittleness of hydroxyapatite. In our study, molecular linker was introduced on the surface of hydroxyapatite microcrystals by surface modification, so that they can participate in polymerization reaction with organic polymer. Those surface-modified hydroxyapatite was named as 'Stickyapatite'. Composite of stickyapatite and organic polymer was constructed on small intestinal submucosa based on layer-by-layer approach, to mimick the hybrid structure of biological bone. In etopic bone marrow model, the hydroxyapatite-chitosan composite showed excellent bone and marrow formation. We expect this sheet-type osteogenic composite construction can be utilized not only for bone regeneration but also for osteo-chondral graft formation. This work was supported by the Basic Science Research Program funded by the Korean Ministry of Science ICT and Future Planning (NRF-2011-0009391) and a grant of the Korean Health Technology R&D Project(HI13C1479), Ministry of Health and Welfane, Republic of Korea.

P852**Board Number: B166****Utilizing Novel Methodologies to Recapitulate the In Vivo Tissue Microenvironment in Cell Culture.**W. Holmes¹, S. Haddad¹, L. Connolly¹, M. Stanton², C. Lambert², R. Bellin¹;¹Biology, College of the Holy Cross, Worcester, MA, ²Chemistry and Biochemistry, Worcester Polytechnic Institute, Worcester, MA

Growing mammalian cells in culture is an invaluable tool for biomedical research. Researchers can study specific effects in a highly controlled environment by growing cells outside of their native tissue. While growing cells in culture has led to the development of significant medical advances, most cells grown in culture do not completely resemble cells in their native tissue. This is often due to the fact that cells are typically cultured on a flat, two-dimensional (2D) dishes, while native tissues are structured in an intricate three-dimensional (3D) arrangement including an extra cellular matrix (ECM). This has led to the development of commercially available 3D cell culture materials, however these are cost-prohibitive and limiting for large scale, high-throughput study

The goal of this project is to develop a novel method of growing cells in culture that mimics the 3D microenvironment of native tissue. In this study, we utilized a polymer of polydimethylsiloxane (PDMS) that is cast on a rough surface, creating a unique textured microenvironment for cells to grow within. Previous results demonstrate that cells grown on these rough PDMS surfaces change their morphology depending on the surface they grow on. Here, we show that these effects are not limited to cell type, but significant morphology changes are observed on three of the most common cell lines used (HeLa, NIH-3T3, and Cos7). It is our hypothesis that these cells will express characteristics similar to that of cells in tissue. To determine this, we compared morphological changes to cells grown on different surfaces, including a 3D matrix (Matrigel). We also assessed changes in the actin cytoskeleton and our results show a significant decrease of actin stress fiber formation on rough PDMS surfaces, similarly seen in cells grown in a 3D matrix. Overall, our studies begin to establish that cells grown on a rough PDMS surface may provide cells with an efficient, cost-effective method to produce a more *in vivo* like environment and allow more findings in cell culture to be translated to the whole organism.

P853**Board Number: B167****Automated Platform for Analysis of Cell-Matrix Adhesions in 2D and 3D Environments.**N. Diggins¹, J. Broussard², S. Hummel³, W. Georgescu^{3,4,5}, V. Quaranta⁶, D. Webb^{1,4,7};

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Cell-extracellular matrix adhesions are of great interest because of their influence on many biological processes, including cell migration, survival, proliferation, differentiation, tissue morphogenesis, wound healing, and tumorigenesis. Adhesions are dynamic structures that have been traditionally characterized on two-dimensional (2D) substrates, although the need to analyze adhesions in more physiologic three-dimensional (3D) environments is being increasingly recognized. However, progress in this area has been greatly hindered by the lack of available tools to analyze adhesions in 3D environments. To address this, we have developed a platform for the automated analysis, segmentation, and tracking of adhesions (PAASTA). This platform enables the quantification of adhesion dynamics and other adhesion characteristics, such as lifetime, location, size, distribution, and shape, in both 3D and 2D environments. We manually validate our platform by analyzing adhesions on 2D substrates and comparing results obtained with PAASTA to traditional analysis methods. Moreover, we use PAASTA to determine rate constants for adhesion assembly and disassembly for cells embedded in 3D type I collagen matrices. Therefore, this platform can be used to analyze multiple characteristics of adhesions and will provide a valuable tool for investigating the molecular mechanisms that regulate adhesion dynamics in 3D environments.

P854**Board Number: B168****Sub-domain FRAP of cell surface molecules to monitor cell surface topography in living cells.**M. Aljumaa¹;¹IMEM, Cardiff University, Cardiff, United Kingdom

It is becoming increasingly clear that many cells have a reservoir of excess plasma membrane in the form of cell surface wrinkles and micro-ridges. The released of these surface structures allow cells to change shape rapidly by permitting an apparent increase in cell surface area eg during phagocytosis or cell spreading. Although indirect methods point to this conclusion, it is only possible to visualise the cell surface topography under scanning electron microscopy and therefore cannot be used to follow surface

topography changes to be monitored in living cells during changes in cell morphology. In this abstract, we describe a novel methodological approach which allows this using the measurement of fluorescence recovery after photobleaching in subdomain at defined 2 dimension distances from the bleach front. Assuming that the diffusion of the fluorescent molecules is constant to rate of recovery at defined points reflects the diffusion distance and hence indicates the surface topography between the bleach front and the measurement domain. Human neutrophils were isolated from blood and their plasma membrane labelled with DiI (DiI C16(3) MWt 877). The dye can be excited using a 543nm laser line and effectively photobleached using the 488nm line transiently elevated. A photo-bleaching zone across the region of the cell of interest was chosen and the image of the cell recording during bleaching and recovery. The bleach/recovery cycle could be repeated. This approach was shown to reflect the cell surface topography by experimentally increasing the wrinkledness of the cell surface using hyperosmotic media. The measured apparent diffusion length increased in the increased wrinkled state and was returned to close to its pre-shrunk value by restoration of isotonicity. The methodology showed that whereas the body of neutrophils has significant ridge-like topographical features, the phagosome and the extending pseudopodia are smooth. This method also showed that elevation of cytosolic Ca²⁺ (by uncaging IP3) influenced the cell surface topography. This methodology opens a novel way of monitoring an important cell characteristic which has not been possible to study previously.

P855

Board Number: B169

Measurement with multi-electrode array system for analysis of temperature-dependent beating rate of cardiomyocytes.

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The beating rate of cardiomyocytes is known to change by environmental temperature. To characterize the beating rate of cardiomyocytes depending on temperature, we measured the beating rate of cardiomyocytes derived from chick embryo (E13) with multi-electrode array (MEA) system. MEA system could be noninvasively measure the extracellular field potential of excitable cells for long-term experiment. The waveforms of the extracellular field potential of cardiomyocytes could be easily detected Na⁺ peak triggered for beating of cardiomyocytes. The interval of between Na⁺ peak and next Na⁺ peak was shown to inter-spike interval (ISI) for the beating rate of cardiomyocytes. The amplitude of Na⁺ peak was shown to the field potential amplitude (FPA) for the activity of Na⁺ channel. Cardiomyocytes cultured on MEA chip were measured with MEA systems after starting the temperature rises from 15°C to 32°C. As a result, the ISI and FPA were recovered synchronous sigmoidal increasing as temperature rises after lag time of several minutes. The stationary phase of the beating rate of cardiomyocytes was constant at a repeated procedure of several times. These results suggested that cardiomyocytes could possess an inherent beating rate corresponding to surrounding environments.

P856**Board Number: B170****Extracellular potential recording of cardiomyocytes derived from chick embryo for drug screening with multi electrode array system.**Y. Shin¹, T. Kaneko²;¹Graduate school of science and engineering, Hosei University, Tokyo, Japan, ²Department of Frontier Bioscience, Hosei University, Tokyo, Japan

Cardiac toxicity testing is essential for drug discovery to consider the effects on the human body in safety pharmacological studies. An electrocardiographic QT interval often extends just before fatal arrhythmia such as torsades de pointes (TdP) caused by the dosage of drugs. A number of drugs are known to cause the electrocardiographic QT interval prolongation by suppressing the rapid delayed rectifier current (IKr), human ether-a-go-go related gene (hERG) channel that functions as a major constituent of K⁺ current. Recently, multi electrode array (MEA) system has been used to measure the field potential duration (FPD) equivalent of QT interval. To examine the availability of MEA system with cardiomyocytes derived from chick embryo (E13) for detect arrhythmogenicity of drugs, we applied K⁺ blocker E-4031 to the cardiomyocytes with this system. As a result, the FPD was extended to 1.2 times compared with control at a final concentration of 10 nM E-4031. Moreover, the Short Term Variability (STV) that is the index of incidence of fatal arrhythmias is increased about 2 times at a final concentration of 10 nM E-4031 and about 10 times at a final concentration of 100 nM E-4031 compared to before addition of drugs. This is the same result in case of cardiomyocytes derived from human ES cells. These results suggested that this MEA system with chick embryonic cardiomyocytes was useful for detection of arrhythmogenicity of drugs. Next, to analyze the drug effect, we applied anticancer drug cisplatin that had been reported having arrhythmia and heart failure as side effects in clinical to this MEA system. There were no effect of cisplatin to the FPD and STV. This result suggested that cisplatin might have no acute toxicity at least for chick embryonic cardiomyocytes.

P857**Board Number: B171****Optimization of medium condition for toxicity testing with multi electrode array system.**Y. Kamei¹, T. Kaneko²;¹Graduate school of science and engineering, Hosei university, Tokyo, Japan, ²Department of Frontier Bioscience, Hosei University, Tokyo, Japan

Multi electrode array (MEA) system is a simple and noninvasive technique to measure the extracellular field potential of excitable cells, and has the possibility to solve the various problems of the toxicity testing. It is known that torsade de pointes (TdP) causes the variation of QT interval on electrocardiogram just before occurring arrhythmia. The variation of QT interval is important to be

detected for the toxicity testing. MEA system could be detected the field potential of cardiomyocytes to analyze the field potential duration (FPD) that equivalent to QT interval and inter spike interval (ISI) that is beating interval. Because its optimum protocol for the toxicity testing is not established, it is necessary to explore medium condition to stably measure the FPD and ISI. In the case of standard Dulbecco's modified Eagle's medium (DMEM), the ISI and FPD often largely vary and are unstable. To stable the pH in the medium, DMEM including HEPES buffer (DMEM with HEPES) and CO₂ independent medium were applied to cardiomyocytes on the MEA chip. We measured the FPD and ISI of cardiomyocytes derived from chick embryo (E13) with MEA system. In the case of pH changes in the medium, DMEM with HEPES was more mildly changed than standard DMEM, and CO₂ independent medium hardly changed. In the waveform of extracellular field potential of cardiomyocytes, the variations of the FPD and ISI were sequentially increased in standard DMEM. However the variations of the FPD and ISI were decreased in DMEM with HEPES and CO₂ independent medium, and the FPD and ISI were stably measured at least for 2 hours. As a result, we found that pH changes in the medium had a negative influence on cardiomyocytes. Therefore, it was suggested that the medium suppressing pH changes could be used for a toxicity testing during several hours.

P858

Board Number: B172

Micro-Cavity based Force Sensors for Cell-biological Studies.

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Mechanics is increasingly recognized as an important factor in numerous biological processes. Monitoring the mechanical properties of cells and tissue is considered a key factor to the understanding of a range of fundamental biological processes and may enable more accurate diagnosis in a range of diseases.

Today, Traction Force Microscopy (TFM) is the most widely-used method to investigate cellular forces exerted on 2D substrates. Until recently, it was assumed that vertical forces can be neglected in the analysis of cell mechanics. However so-called 2.5D TFM studies have now shown that the out-of-plane and in-plane forces exerted by cells are of the same order of magnitude. Although 2.5D TFM has contributed to our understanding of vertical forces for different cell types, it suffers from several immanent limitations: The need to perform 3D confocal laser scanning for bead tracking and associated photo-toxic effects limit temporal resolution and restrict the field of view when performing time-lapse measurements. The need to take zero-force images at the end of each experiment complicates the measurement further.

Here, we present a completely new approach to measure cellular forces which overcomes the limitations of 2.5D TFM by interferometrically detecting deformations of an elastic probe rather than by tracking bead displacement. The centrepiece of our innovation is a novel optical micro-cavity sensor that enables fast force mapping across a large field of view by analysing changes in resonance wavelength.

Our approach avoids any phototoxic effects and therefore allows the measurement of cellular forces at high frame rates over hours or days. Being based on wide-field imaging, our new method measures the deformation at each point of the image simultaneously and with diffraction limited lateral resolution. Vertical displacements are detected with accuracy far beyond conventional confocal microscopy (5 nm or better). Force maps can be recorded without the need for zero-force images, increasing throughput, eliminating the need to detach non-migrating cells after force mapping and allowing measurements of multiple cells on one substrate. Additionally, the optics needed for the readout of the new biosensor can be readily integrated with a conventional inverted microscope.

In this presentation we will discuss the fabrication of our micro-cavity sensors and provide detailed investigations of force and spatial resolution of the device by means of AFM indentation analysis. Cell mechanical measurements of different cell lines, including human neuronal cells and primary immune cells, will be presented and links between recorded force patterns and subcellular structures labelled by fluorescence staining will be discussed.

P859

Board Number: B173

Nanoparticles and the blood-brain barriers in vitro: Carriers as well as barrier modulators.

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Nanoparticles are widely used in different areas as food, cosmetics, surface protective in clothes and biomedical products. Moreover they are proposed to be useful carriers to overcome biological barriers. But besides the widespread use and favorable characteristics of nanoparticles (NP) there has been some concern about their biological safety. New techniques have to be emerged to fast and reliably determine possible interferences with barrier properties. The electrical resistance together with the electrical capacitance is such a useful technique In vivo studies in rats have shown that e.g. silver-NPs accumulate in various organs including the brain after both oral exposure and inhalation. Since the mechanism of brain transfer is not known it is important to study the effects of Ag-NPs on the barriers of the central nervous system, namely the blood-brain barrier (BBB) and the blood-cerebrospinal fluid (CSF) barrier. The aim of the present in vitro study was to analyze the effects of two differently modified Ag NPs (Ethylene oxide [EO] and Citrate) on primary porcine brain capillary endothelial cells (PBCEC) and, for the first time, on primary capillary choroid plexus epithelial cells (PCPEC) (Cramer et al 2014) The effect to weaken the barrier may be used for drug targeting to the brain if barrier opening occurs in a given short time window and is reversible. This will be demonstrated by the use of poly-butylcyanoacrylate-NPs. We will show that these nanoparticles open the blood-brain-barrier in a time window of about 2 hours which allows a drastic flux of even larger molecules by passive diffusion through the tight junctions (Rempe et al 2011) Here we will present the useful application of a new technique called Impedance spectroscopy to investigate barrier properties of cells grown on permeable filters to quantify the barrier

properties. Correlation to permeability changes and cellular responses like cytotoxicity, oxidative stress and inflammation will be shown.

P860

Board Number: B174

3D isotropic volume imaging and reconstruction of biological samples using SEM.

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In recent years there has been a considerable advancement in SEM-based methods for 3D reconstruction of large tissue volumes. Serial Block-Face SEM (SBF-SEM) involves combination of imaging and in-situ sectioning of plastic embedded tissue blocks within the SEM vacuum chamber, allowing for automated imaging and subsequent reconstruction of volumes of tissue. With a novel solution for high spatial resolution and throughput SEM volume imaging overcoming the resolution limits set by mechanical slicing by combining it with virtual sectioning. Virtual slicing is realized by Multi-Energy Deconvolution SEM (MED-SEM), a non-destructive technique that allows high resolution reconstruction of the top layers of the sample. This cycle of physical and virtual sectioning offers isotropic datasets with excellent z-resolution and can be fully integrated and automated. For experiments where even greater isotropic resolution is needed, DualBeam systems excel offering up to 3nm isotropic resolution in 3D volume dataset. DualBeam scanning electron microscopes are equipped with both ion- (FIB) and electron-beam (SEM) and offer a new solution to explore subsurface structure of biological tissues. It not only provides high resolution SEM imaging, but also enables precise cross sectioning or slicing with the FIB on selected positions on the sample. With the new advanced detection technologies available, low kV imaging enables the acquisition of series of images of the tissue block face without charging. Using the automated software solutions, the acquisition of the high quality 3D dataset needs minimal user intervention, and so offers instant and high yield productivity. Furthermore the potential combination of the 3D imaging systems with fluorescence microscopes opens new doors for site specific structural analysis. In this work we demonstrate the process what the eukaryotic cells using to eliminate foreign object from themselves. The details of the endocytotic pathway are revealed in a live and dynamic environment using fluorescently labelled foreign objects. Then using correlative light and electron microscopy approach the region of interest was located. Then the lysosome structure with the fluorescence foreign object was examined in a 3D with high resolution details using scanning electron microscopy.

P861**Board Number: B175****Intracellular delivery by cell squeezing is affected by perturbation of actin cytoskeleton and lipid rafts.**M.P. Stewart¹, X. Ding¹, K. Jensen², A. Sharei¹, R.S. Langer¹;¹Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA,²Chem Eng, Massachusetts Institute of Technology, Cambridge, MA

Cell squeezing is a new intracellular delivery modality based on rapid mechanical deformation of cells passaging through precisely fabricated constrictions in a microfluidic device. Delivery of molecules is achieved by diffusion through membrane disruptions, however, little is known about how the properties of the cell surface affect the membrane disruption and recovery behavior. Using specific inhibitors in conjunction with flow cytometry to assess delivery efficiency, we uncover roles for actin and lipid rafts in the cell response. While perturbation of rafts decreased cell robustness and recovery, inhibitors of actin dynamics exhibited variable effects, with some even improving membrane recovery. We anticipate continued studies on these underlying mechanisms should yield insight into the mechanical response to rapid cell deformations and lead to improved efficiency in delivery applications.

P862**Board Number: B176****Why only stretch when you can also see? Correlative optical Tweezers-Fluorescence Microscopy (CTFM) as a versatile tool for cell biology.§.**A. Candelli^{1,2}, J. Block³, I. Brouwer¹, I. Heller¹, G. Sitters^{1,2}, O.D. Broekmans¹, M. Modesti⁴, S. Köster³, E.J. Peterman^{1,2}, O.T. Heyning², G.J. Wuite^{1,2};¹Department of Physics and Astronomy, VU University Amsterdam, Amsterdam, Netherlands, ²LUMICKS bv, Amsterdam, Netherlands, ³Georg-August-Univ Gottingen, Gottingen, Germany, ⁴Institut National de la Santé et de la Recherche Médicale, Marseille, France

Correlative optical Tweezers-Fluorescence Microscopy (CTFM), combines optical tweezers with confocal fluorescence and STED nanoscopy. CTFM is a promising approach for the investigation of biological systems, because it uniquely allows to simultaneously manipulate, sense and visualize individual molecules with exquisite sensitivity and resolution. Here we present our efforts to broaden the applicability of CTFM technology by developing an integrated instrument that allows turn-key operation with ease of use, without compromising performance. Using this instrument, we carry out a series of demonstration experiments, exploring applications of CTFM in DNA-protein interactions, nano-mechanics of intermediate filaments, synthetic models of signal transduction, membrane fusion and molecular motors. These experiments show that the technological advances in hybrid single-molecule methods allow the development of an easy-to-use and stable instrument that can be applied in many research areas and operated by researchers with diverse training and background.

P863**Board Number: B177****Cell proliferation studies with a bioadhesive hydrogel for intervertebral disc tissue regeneration.**A. Nitzl¹, E. England¹, T. Christiani^{1,2}, J. Vernengo^{1,2}, J. Kadlowec^{1,3}, C. Iftode¹;¹Biological Sciences, Rowan University, Glassboro, NJ, ²Chemical Engineering, Rowan University, Glassboro, NJ, ³Mechanical Engineering, Rowan University, Glassboro, NJ

The goal of our research is to develop a three-dimensional culture system for use in intervertebral disc tissue engineering. As such, thermosensitive biomimetic hydrogels will be designed to support long-term viability and differentiation of adipose stem cells towards the nucleus pulposus (NP) phenotype. Our first objective is to produce a polymeric scaffold with superior mechanical strength and bioadhesive properties, while remaining biocompatible with the stem cells it envelopes. Previously, we reported on the cytoviability of a base polymer consisting of 5% poly-N-isopropylacrylamide grafted chondroitin sulfate (PNIPAAm-g-CS). In our current work, we present cell studies performed with a new bioadhesive scaffold synthesized by blending alginate microparticles with PNIPAAm-g-CS. The new polymer was designed to achieve increased bioadhesive properties, a crucial requirement for prevention of dislocation of intervertebral disc implants. Both qualitative (LiveDead) and quantitative (XTT) viability tests performed with this new composite polymer show that it supports HEK-293 and adipose-derived stem cell (ASC) survival to approximately 85% of that of PNIPAAm-g-CS alone. In preliminary studies, we have also found that increasing the alginate particle size (> 89.8 microns) doubles polymer tensile strength and enhances cell proliferation of HEK-293 cells in direct comparison with particles sizes less than 16 microns. Future work includes differentiation of ASCs in the PNIPAAm-g-CS-alginate polymer toward the NP phenotype and confirmation NP marker expression by immunohistochemistry and RT-PCR.

P864**Board Number: B178****Novel particles for transportation of active proteins into cellular nuclei.**C.L. Minchew^{1,2}, V.V. Didenko^{1,2};¹Baylor Coll Med, Houston, TX, ²Michael E. DeBakey Veterans Affairs Medical Center, Houston, TX

We describe a new type of multifunctional fluorescent nanoparticle for the targeted induction of cell death in the specified cell types, such as cancer cells. The particles employ a novel internal architecture and are structured assemblies of fusion proteins and cationic polymers. They have interchangeable targeting moieties with the ability to be re-targeted to a variety of cell types. After injection into the tumor site, they can penetrate tumor cell membranes and selectively passage into their nuclei. Being fluorescent, the new nanoparticles can simultaneously signal the response to the therapy. The new

development is applicable in anticancer research which uses cell-specific delivery and fluorescence imaging.

P865

Board Number: B179

Reversible protein inactivation by optogenetic trapping in cells.

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The precise inhibition of target proteins can serve as an effective means to dissect complex signaling networks or to identify new therapeutic targets. Here we present a versatile platform to inactivate proteins in living cells using light, light-activated reversible inhibition by assembled trap (LARIAT), which sequesters target proteins into complexes formed by multimeric proteins and a blue light-mediated heterodimerization module. Using LARIAT, we inhibited diverse proteins that modulate cytoskeleton, lipid signaling and cell cycle with high spatiotemporal resolution. Use of single-domain antibodies extends the method to target proteins containing specific epitopes, including GFP.

P866

Board Number: B180

Cas9 driven by an optimal promoter improves gene editing in eukaryotic cell lines when paired with synthetic crRNA and tracrRNA.

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Genetic engineering of living cells is of critical importance to understanding how genes influence organisms. Recently, the CRISPR-Cas9 system has gained popularity in the gene editing field due to its relative elegance and ease of use compared to other gene editing methods. This system requires a complex of the Cas9 protein with a gene-targeting CRISPR RNA (crRNA) and a trans-activating RNA (tracrRNA), to introduce DNA breaks at specific locations in the genome in a single- or multi-gene fashion.

Presented here are results on the efficiency of using synthetic crRNA and tracrRNA to introduce gene editing events when co-transfected with a plasmid expressing Cas9. Expressing humanized Cas9 from different promoters (e.g., human and mouse CMV and EF1alpha, PGK, CAG), along with synthetic crRNA and tracrRNA, results in varying amounts of gene editing in different cell lines and cell types. The efficiency of gene editing can be improved with the use of the optimal promoter in chosen cell lines, demonstrating the importance of choosing the most active promoter in the cell line of interest.

Additionally, the Cas9 gene can be expressed with either a puromycin selection marker or a red fluorescent protein for FAC sorting to allow enrichment of transfected cells. Both methods result in enrichment of cells with gene editing events by at least two-fold as measured by a mismatch-specific nuclease assay. Importantly, results demonstrate that the use of synthetic crRNA and tracrRNA offers a simplified and faster method for gene editing of one or more genes without requiring any cloning steps. By virtue of its simplicity, this three-component CRISPR-Cas9 system is amenable to high-throughput genome editing applications.

Actin and Actin-Associated Proteins 2

P867

Board Number: B182

Integration of Linear and Dendritic Actin Nucleation in Nck-Induced Actin Comets.

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We propose that the Nck adaptor protein can serve as an integrator of linear and branched nucleation in actin comet tails. In our studies we characterize comet tails induced by experimental aggregation of Nck-SH3 domains at the membrane. They are extremely similar in their shape and dynamics to *Vaccinia* comets and serve as a good model to study the role of Nck adaptor in signaling to the actin cytoskeleton and maintenance of the comet tail phenotype. Experimentally shifting the tight unbranched/branched nucleation balance altered morphology and dynamics of Nck-SH3-induced actin comets. Inhibition of linear formin-based nucleation with the small molecule inhibitor SMIFH2 resulted in formation of predominantly circular actin structures with decreased mobility. The proline-rich FH1 domain of formins recognizes Src-homology 3 (SH3) domains. We overexpressed FH1 domain to inhibit the binding of endogenous full-length formin on Nck-SH3-induced actin comets. As a result, cells produced less dynamic and mostly circular actin structures. Additionally, enhancement of branched Arp2/3-mediated nucleation, caused by N-WASP overexpression, similarly caused loss of the typical actin comet tail shape. These results indicate that formin-based linear actin polymerization is critical for the formation of Nck-dependent actin comet tails and maintenance of their phenotype. In fact aggregation of only branched active Nucleation Promoting Factor (VCA domain of N-WASP) with the density and turnover similar to that of N-WASP in Nck comets, does not reconstitute dynamic elongated actin comets. The ratio of linear to dendritic nucleation activity may, thus, serve to distinguish the actin structures induced by viral and bacterial pathogens.

P868**Board Number: B183****Cordon Bleu serves as a platform at the basal region of microvilli where it regulates microvillar length through its WH2 domains.**J. Wayt¹, A.P. Bretscher¹;¹Cornell University, Ithaca, NY

Cordon Bleu (Cobl) is a WH2-containing protein believed to act as an actin nucleator. We show that it has a very specific localization in epithelial cells at the basal region of microvilli, a localization unlikely to be involved in actin nucleation. The protein is localized by a central region between the N-terminal COBL domain and the three C-terminal WH2 domains. Ectopic expression of Cobl shortens apical microvilli, and this requires functional WH2 domains. Proteomic studies reveal that the COBL domain binds several BAR-containing proteins, including SNX9, PACSIN-2/Syndapin-2 and ASAP1. ASAP1 is recruited to the base of microvilli by binding the COBL domain through its SH3. We propose that Cobl is localized to the basal region of microvilli to participate in both length regulation and to recruit BAR proteins that associate with the curved membrane found at the microvillar base.

P869**Board Number: B184****A coupled in vitro-in vivo approach to dissect APC-Diaphanous mediated actin assembly.**O. Molinar¹, R. Jaiswal², E. Kunttas-Tatli¹, A. Rankova², V. Stepanik¹, B.L. Goode², B.M. McCartney¹;¹Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA, ²Department of Biology, Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, MA

The multifunctional Adenomatous polyposis coli (APC) proteins are known for their roles in negatively regulating Wnt signaling, stabilizing microtubules, and indirectly regulating actin through effectors such as Asef and IQGAP. Most recently, we have shown that through their basic domains both vertebrate APC (vAPC) and *Drosophila* APC1 can bundle and nucleate actin filaments, and collaborate with the formin Diaphanous (Dia) to efficiently nucleate actin assembly *in vitro*. In addition, *Drosophila* APC2 (which lacks a basic domain) and Dia bind directly and are required for actin furrow extension in the fly embryo. APC1 does not function in actin furrow extension. Together these data suggest that APC-Dia collaborations are an evolutionary conserved mode of actin filament assembly regulation. However, significant gaps exist in our understanding of both mechanism and physiological relevance. While we have investigated the mechanism underlying the vAPC/APC1-Dia collaboration in detail, how APC2 affects Dia activity without a basic domain is not known. Here we demonstrate that APC2 interacts with Dia through its β -catenin binding 20 amino acid repeats (20Rs), and that 20R phosphorylation by GSK3 β regulates APC2's actin furrow activity. Contrary to our predictions that APC2 promotes Dia's actin assembly activity in the embryo, APC2 specifically inhibits the actin assembly activity of both APC1 and

Dia in vitro. We are currently working to reconcile these results and develop a deeper mechanistic understanding of APC2-APC1-Dia interactions. As a complement to these analyses, we are dissecting the role of APC-Dia collaborations *in vivo*. Many coordinated actin-mediated processes occur during *Drosophila* oogenesis making this a great physiologically relevant system in which to study actin assembly. In stage 10B egg chambers, an array of cytoplasmic actin bundles (actin baskets) form around each nucleus in the nurse cells to secure them. Loss of these baskets disrupts oogenesis resulting in small, round, inviable eggs. Here we show that APC1 and Dia are required for the proper assembly of these actin baskets. In *APC1* or *dia* mutants, the actin baskets fail to form at stage 10B, but surprisingly they are present at stage 11. This delay may suggest that APC1-Dia function can be compensated for by other assembly factors. Alternatively, APC1 and Dia may be able to carry out actin assembly alone in the absence of the other partner, but with reduced efficiency resulting in assembly delay. In conclusion, the *Drosophila* APC-Dia collaborations represent an evolutionarily conserved, experimentally tractable system in which to dissect both molecular mechanisms and physiological relevance.

P870

Board Number: B185

Calponin-like protein isoforms regulate actomyosin contractility in *C. elegans*.

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Calponins and calponin-related proteins are present widely in eukaryotes and regulate stability of actin filaments and contractility of actin and myosin. Although a number of biochemical studies suggest their regulatory roles for actin cytoskeleton, little is known about their *in vivo* functions. In mammals, calponins and calponin-related proteins have a calponin-homology (CH) domain at the N-terminus and one or three calponin-like (CLIK) motifs at the C-terminus. In the nematode *Caenorhabditis elegans*, UNC-87 is a unique calponin-related protein with seven CLIK motifs and no CH domain and has been shown previously to stabilize actin filaments in striated muscle (Goetinck and Waterston, 1994; Yamashiro et al., 2007). Here, we report that multiple isoforms of calponin-like proteins are expressed in *C. elegans* and have functions to regulate actomyosin contractility in striated and non-striated muscles. The *unc-87* gene has two alternative first exons, which are used to generate two UNC-87 isoforms (Goetinck and Waterston, 1994): UNC-87A with a longer N-terminal sequence and UNC-87B with a shorter N-terminal sequence. A promoter-reporter analysis showed that UNC-87A and UNC-87B are expressed in different tissues. UNC-87A was expressed in pharynx, uterine muscle, and neurons, whereas UNC-87B was expressed in body wall muscle and the somatic gonad. *In vitro*, UNC-87A and UNC-87B bound to actin filaments and bundled them. They also bound to myosin and inhibited actin-activated myosin ATPase. Both UNC-87A and UNC-87B induced large actomyosin bundles that are resistant to ATP-induced disassembly. In these *in vitro* experiments, UNC-87A exhibited stronger binding to actin and myosin than UNC-87B, suggesting that these isoforms have different regulatory functions for actin and myosin. In addition, *C. elegans* has another gene *clik-1*, which encodes a protein with seven CLIK motifs and a 46 % identical sequence to UNC-87B. Although a single knockout of *clik-1* did not cause

any detectable phenotypes, knockdown of *clik-1* in *unc-87* mutants caused paralysis and defective egg laying. Simultaneous depletion of *unc-87* and *clik-1* also caused excessive contraction of the myoepithelial sheath in the somatic gonad, suggesting that they have overlapping functions to inhibit actomyosin contractility. These results suggest biological significance of calponin-related proteins with CLIK motifs in actomyosin regulation.

P871

Board Number: B186

The actin crosslinking protein palladin modulates force generation and mechanical sensing by cells.

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Pancreatic tumor-associated fibroblasts (TAFs) have been shown to promote the progression of pancreatic tumors, metastasis, and the resistance to therapy. Mechanisms by which these cells stimulate invasiveness and metastasis of cancer cells are not well understood. It is known that the actin-crosslinking protein, palladin, is upregulated in TAFs, but the precise role of palladin in these cells is unknown. Several lines of evidence suggest that the misregulation of actin reorganization resulting from enhanced palladin levels may contribute to aberrant cellular behavior. In this study we use human pancreatic tumor associated fibroblasts (TAFs) to investigate the role of palladin in regulating the plasticity of the actin cytoskeleton and cellular force generation in response to alterations in substrate stiffness. Traction force microscopy revealed that TAFs are sensitive to substrate stiffness as they generate larger forces on substrates of increased stiffness. Contrary to expectations, knocking down palladin increased the forces generated by cells accompanied by significant differences in the actin organization palladin knock down cells. Our results suggest that the actin crosslinkers such as palladin and myosin motors coordinate for optimal cell function and to prevent aberrant behavior as in cancer metastasis.

P872

Board Number: B187

Calcium-regulated actin bundling activity of human plastins.

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The actin cytoskeleton is an extensive and intricate network that is tightly regulated by a vast array of actin binding proteins. Plastins are one such family of proteins that are conserved throughout eukaryotic

life with three isoforms expressed in mammals. Each of these isoforms is tissue-specific: plastin 1 (PLS1) is expressed in the intestines and kidneys, plastin 2 (PLS2) is expressed in hematopoietic cells and malignant tumors, while the most abundant plastin 3 (PLS3) is present in solid tissues. The three isoforms are involved in distinct functions including epithelium strengthening, cell migration, and differentiation. Plastins crosslink actin filaments into tight bundles essential for cellular structures such as microvilli, lamellipodia, and filopodia. This bundling is implemented by two tandem actin binding domains (ABDs) and regulated by an N-terminal domain containing two EF hand motifs. Binding of Ca^{2+} to the EF hands inhibits the bundling activity of all isoforms, however, a detailed molecular mechanism of this inhibition is not understood. Recently, it has been reported that the isolated EF-hands of PLS3 have two different affinities to Ca^{2+} (low $K_d \sim 10 \mu\text{M}$ and high $K_d \sim 0.4 \mu\text{M}$); whereas both EF-hands of PLS2 bind Ca^{2+} with similar high affinity ($K_d \sim 1 \mu\text{M}$). Based on this observation it has been proposed that PLS3 is more suitable for maintaining static cytoskeleton, while PLS2 is more suitable for dynamic rearrangements characteristic for actively migrating immune cells. In the present study we readdress this subject by using full-length proteins and monitoring functional effects of Ca^{2+} interaction with plastin. To this end, we employed light scattering to directly monitor Ca^{2+} -induced disassembly of actin bundles formed by the three plastin isoforms. Phosphorylation of Ser5 on PLS2 has been shown to dramatically improve its binding to actin. Accordingly, only the phosphomimetic mutant of PLS2, but not the wild-type protein, bundled actin in our assays. Intriguingly, we found that all three isoforms of recombinant human plastins have similar sensitivity to Ca^{2+} within the physiologically relevant pCa 6.3-6.5. While efficiently dismantling actin bundles, Ca^{2+} did not substantially affect binding of any of the three isoforms to actin suggesting that binding of only one of the domains (ABD1 or ABD2) is inhibited by EF hands. Interestingly, a construct of PLS3 lacking the C-terminal actin binding domain (ΔABD2) bound actin in Ca^{2+} -independent manner with an affinity similar to that of full-length PLS3. This result indirectly implies that binding of ABD2 rather than ABD1 is inhibited by the EF hands. Work is ongoing to further characterize the effect of Ca^{2+} on plastin binding to filamentous actin and to decipher a detailed Ca^{2+} regulatory mechanism.

P873

Board Number: B188

Regulation of macrophage motility, morphology and activation by the actin-bundling protein L-plastin.

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Macrophage motility requires precise spatiotemporal control of actin dynamics. The actin-bundling protein L-plastin (LPL; also called fimbrin) is highly enriched in macrophage podosomes. Essential functions for LPL in macrophage biology have not yet been fully described. Prior work has demonstrated reduced motility and transmigration in lymphocytes lacking LPL. Investigation of the cellular mechanisms underlying the increased susceptibility of mice genetically deficient for LPL (LPL^{-/-} mice) to pulmonary

infection with the bacteria *Streptococcus pneumoniae* revealed a developmental defect in alveolar macrophages. Alveolar macrophages are a tissue-specific, developmentally unique population of phagocytes critical to normal lung innate immunity. Building upon these prior observations, we now report the requirement for LPL in macrophage morphology, motility, and activation. Alveolar macrophages derived from LPL^{-/-} mice demonstrated reduced spreading on glass coverslips compared to macrophages sufficient for LPL. Actin recruitment to sites of vinculin aggregation was impaired in LPL^{-/-} alveolar macrophages, revealing impaired podosome formation. Peritoneal macrophages from LPL^{-/-} mice were defective in transmigration across collagen-coated Transwell inserts. These morphologic and motility defects correlated with activation defects in LPL^{-/-} alveolar macrophages. Using the NLRP3 inflammasome activator ATP after LPS priming, we demonstrated reduced production of IL-1-beta by LPL^{-/-} alveolar macrophages. IL-6 production was also reduced but secretion of TNF-alpha was unimpaired, suggesting selective activation defects in the absence of LPL. The mechanistic link between disruption of cytoskeletal regulation and NLRP3 inflammasome activation in cells lacking LPL is an area of active investigation. Transcripts of IL-1-beta were reduced in LPL^{-/-} alveolar macrophages, suggesting that LPL may be required to maintain cells in a "readiness" state to receive and respond appropriately to activation signals. Further investigation of macrophages from LPL^{-/-} mice will reveal novel insights into the molecular mechanism by which actin cytoskeletal elements regulate immune cell activation.

P874

Board Number: B189

The function and dynamics of the apical scaffolding protein E3KARP is regulated by cell cycle phosphorylation.

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Scaffolding proteins, often containing PDZ domains, bring together other proteins to facilitate their collective activity. Here we examine the apical protein E3KARP/NHERF2 and find that its function and dynamics is cell cycle regulated, but in a mechanistically different manner from its paralog EBP50/NHERF1. The identification of novel phospho regulatory sites has allowed us to investigate their functional significance. Phosphorylation at serine 303 in the tail region of E3KARP greatly enhances the in vivo dynamics of E3KARP in cells during mitosis. Phosphomimetic S303D enhances the dynamics and compromises the ability of E3KARP to complement the function of EBP50 in generating microvilli on interphase cells. S303D enhances the dynamics of the isolated E3KARP tail in vivo, and, in contrast to EBP50, the presence of the PDZ domains does not regulate the dynamics. However, in vitro, the interaction of E3KARP with active ezrin is unaffected by S303D, indicating the involvement of another factor that enhances in vivo dynamics. Inhibitor, knockdown and localization studies indicate that A-Raf is required for S303 phosphorylation in mitotic cells. Interestingly, in both cases the mechanisms regulating dynamics involve the tails, which are the most diverged region of the paralogs, and probably evolved independently after a gene duplication event that occurred early in vertebrate evolution.

P875**Board Number: B190****Septin 9 is an actin binding protein which bundles F-actin through a unique N-terminal domain.**C. Smith¹, R. Van Sciver¹, L. Dolat², E.T. Spiliotis², V.E. Galkin¹;¹Eastern Virginia Medical School, Norfolk, VA, ²Biology, Drexel University, Philadelphia, PA

Septins are GTP-binding proteins that are crucial for cell division and nervous system development. They are structurally related to the Ras GTPases but unlike the monomeric small GTPases, septins readily polymerize into filaments, bundles and rings comprised of tissue specific septin isoforms. SEPT9 is a unique septin that possess an N-terminal extension (NTE) domain that is missing in the other septin isoforms. Recently it has been shown that septins interact with both actin filaments and microtubules. *Drosophila* septin complexes form curved bundles out of actin filaments, while human SEPT9 is capable of promoting bundle formation out of both microtubules and F-actin. We used electron microscopy and image analysis to show that SEPT9 bundles F-actin via its NTE, and that the basic domain of the NTE is responsible for the actin bundling. We also show that GTP-binding domain (G-domain) of SEPT9 readily binds but does not bundle F-actin. Both the NTE-domain and the G-domain may compete with the NTE for the same binding site of the F-actin surface. Our structural data indicate that SEPT9 might directly affect acto-myosin contractility and participate in regulation of cell motility.

P876**Board Number: B191****Structural Basis of the Vinculin–F-actin Interaction.**L.Y. Kim¹, P. Thompson², S. Campbell², G.M. Alushin¹;¹CBPC, NIH/NHLBI, Bethesda, MD, ²Biochemistry and Biophysics, UNC, Chapel Hill, NC

The essential protein vinculin is a key component of cell–matrix and cell–cell adhesions, acting as a linker between these connection points and the actin cytoskeleton. Accumulation of vinculin is necessary for adhesions to sustain high traction forces, and its direct interaction with F-actin is required for mechanotransduction. Due to its physiological significance, the molecular determinants of this interface have been under scrutiny for some time, yet conflicting structural models are present in the literature, largely because of the absence of high-resolution analysis. We have obtained a subnanometer-resolution (8.5 Å, gold-standard FSC 0.143) cryo-EM reconstruction of vinculin’s C-terminal “tail” domain (Vt) bound to F-actin, sufficient for constructing an unambiguous pseudo-atomic model of the interface by flexible docking of crystallographic structures of the components. Our findings support a recent experimentally derived model of the interaction (Thompson et al., *Structure* 2014). Additionally, we find that Vt undergoes a substantial conformational change upon actin binding, characterized by a twisting of helices 4 and 5 and unfolding of helix 1 from the bundle, a segment of which makes a novel contact with the surface of the actin filament. We postulate that this structural

transition enables additional interactions between vinculin and binding partners upon actin engagement.

P877

Board Number: B192

Metavinculin alters the mechanical properties of F-actin and attenuates the bundling activity of vinculin.

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Metavinculin is a longer and muscle specific splice variant of vinculin. Despite the link between mutations in the tail of metavinculin (MVT) and human cardiac disease, the function of MVT remains elusive. Recent work proposed that MVT severs F-actin. Therefore, we characterized this activity and the structural changes in F-actin that manifest upon MVT binding. Using mutagenesis, site-directed fluorescent labelling and TIRF microscopy, we found that MVT alters the actin arrangement of actin interprotomer contacts and fragments F-actin most potently at half binding densities. However, we did not observe MVT's severing activity under real time conditions. This raises the possibility that the fragmentation observed in our equilibrium assays may result from an MVT induced change in F-actin bending mechanics. Consequently, we assessed the flexibility of F-actin decorated with MVT by measuring the bending persistence length. Indeed, we observed a difference. Finally, we explored the interplay between VT and MVT by monitoring the F-actin bundle formation using TIRF microscopy. Our results on MVT induced changes in actin filament flexibility and altered actin filament organization will be presented.

P878

Board Number: B193

Cyclase Associated Protein Regulates Alpha-Actinin Function in Epithelia.

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Alpha-Actinin is a well-known actin binding and bundling protein. It has been shown to localize to many known actin structures including stress fibers, adherens junctions, lamellipodia, and the cortical actin cytoskeleton. Understanding the actinin interactome will help us better understand its role in and regulation of each of the structures it localizes to. With this in mind, we performed a pull down of kidney extract using recombinant actinin as bait. The main hit from our pull down was Cyclase

Associated Protein 1, CAP1. CAP1 has been shown in our lab as well as others to facilitate depolymerization of actin filaments in vitro and to both increase and decrease the amount of actin in different actin structures in cells by as yet unknown mechanisms. In MDCK cells, a canine kidney epithelia cell line, CAP1 was localized to cell-cell borders, actin protrusions, and basal fibers. Actinin also localizes to these structures, however, when we examined the localization of the two proteins along stress fibers they were predominantly not colocalized. We then knockdown CAP1 in MDCK cells and found that the actin signal at the basal surface increased as well as the actinin signal. This is consistent with a new model where CAP1 either sequesters or buffers the amount of actinin to modulate stress fibers. We are currently testing this model further with in vitro biochemistry.

P879

Board Number: B194

Quantitative comparison of human Cofilin-1, Cofilin-2, and ADF effects on actin dynamics.

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Cofilin/Actin-Depolymerizing Factor (ADF) family proteins bind to actin filaments and actin monomers and perform essential in vivo roles in driving the dynamic remodeling and turnover of the actin cytoskeleton. Mammals express three different Cofilins: Cofilin-1, Cofilin-2, and ADF. Cofilin-1 and ADF are more ubiquitous and often co-expressed in the same cell types, whereas Cofilin-2 is muscle-specific. Here, we performed a direct and quantitative comparison of the activities of all three human Cofilins on actin filament and monomer dynamics in vitro using both TIRF microscopy and bulk fluorescence assays. Our results show that all three Cofilins bind actin monomers with similar affinities ($K_d \sim 1.3 \mu\text{M}$) and block nucleotide exchange. However, Cofilin-2 and ADF sever filaments with higher efficiency than Cofilin-1. Further, in the presence of Aip1 and Cor1B, Cofilin-2 and ADF disassembled filaments more efficiently than Cofilin-1. We have also engineered cysteine residues in each Cofilin to generate fluorescently labelled functional molecules for multi-wavelength TIRF experiments aimed at directly visualizing and comparing the interactions of each Cofilin with actin filaments. Finally, we have begun comparing the activities of each Cofilin on branched versus bundled actin filament networks.

P880**Board Number: B195****GMF β controls branched actin content and lamellipodial retraction in fibroblasts.**E.M. Haynes¹, S.B. Asokan¹, S.J. King¹, C. Wu^{1,2}, I.P. Lebedeva^{1,2}, J.E. Bear¹;¹UNC Lineberger Comprehensive Cancer Center, Department of Cell Biology and Physiology, University of North Carolina, Chapel Hill, NC, ²Howard Hughes Medical Institute, Chevy Chase, MD

The process of cell migration is integral to both organism development and disease. In order to migrate, cells rely on the lamellipodium, a fan-like structure comprised of Arp2/3 nucleated branched actin which generates the force necessary for leading edge protrusion. Polymerization of actin at the leading edge is relatively well understood; however, much less is known about branched actin disassembly/remodeling and how it influences migration. In order to respond to extracellular cues, a cell must be able to quickly remodel its actin cytoskeleton, a process which relies on actin severing and pruning of branches. The cofilin homolog GMF has been implicated in disassembly of branched actin mainly by in vitro work. We sought to understand the role of the ubiquitous mammalian GMF isoform, GMF β , in lamellipodial dynamics and cell migration. We found that GMF β (but not GMF γ) is expressed in fibroblasts and localizes to the leading edge of the cell. Modulation of GMF β by depletion or overexpression resulted in significant phenotypes in lamellipodial dynamics, branched actin content and migration. Specifically, depletion of GMF β resulted in larger lamellipodia with reduced ability to retract, and a higher fraction of the cell periphery positive for Arp2/3. GMF β overexpressing cells were smaller and had a reduced fraction of Arp2/3 positive cell periphery. These data are consistent with the notion of GMF β as an antagonist of branched actin, but does not differentiate between GMF β 's two proposed mechanisms of action: debranching and preventing nucleation promoting factors from binding to Arp2/3. In order to address whether branch lifetime itself was affected by GMF β , we performed a wash-in using the Arp2/3 inhibitor CK666. Based on previous studies (Hetrick et al 2013) CK666 binds free Arp2/3 and prevents formation of new branches, but branches that are already formed will not disassemble in the presence of CK666. GMF β depletion increased the time required for Arp2/3 signal to dissipate from the cell edge after CK666 wash-in, suggesting that GMF β is important for debranching. In addition, we created a mutation in GMF β analogous to a mutation in budding yeast GMF (Ydenberg et al 2013) which severely reduces debranching activity. This mutant cannot rescue the observed phenotypes of depletion of wild type GMF β . Finally, we tested if the changes we observed in the branched actin content in these cells affected directional migration. While both GMF β depleted and GMF β overexpressing cells could detect a gradient of soluble cue (chemotaxis), we found that both cell lines were unable to detect a gradient of bound extracellular matrix (haptotaxis). This corroborates previous results from our lab that the branched actin generated by Arp2/3 is necessary for haptotaxis.

P881**Board Number: B200****Sid2 phosphoregulation of anillin-related Mid1 nuclear localization and fission yeast cytokinesis.**A.K. DeWitt¹, P. Schneider¹, G. Foa¹, K.L. Gould², D.M. Clifford Hart¹;¹Cell and Molecular Biology, Grand Valley State University, Allendale, MI, ²Cell and Developmental Biology, Vanderbilt Univ Med Sch/HHMI, Nashville, TN

Cortical nodes assemble during interphase and serve as the foundation to generate a contractile ring for fission yeast cytokinesis. The anillin-related protein Mid1 serves as a central organizer of cytokinesis by anchoring cortical nodes to the cell membrane and recruiting proteins that incorporate into the contractile ring. Interestingly, Mid1 dissociates from the cell cortex prior to contractile ring constriction. However, it remains unclear if Mid1 departure influences ring function. Given that localization of Mid1 to the cell cortex is phosphorylation dependent, we hypothesized that Mid1 departure from the cell cortex may be similarly controlled. Indeed we find that Sid2 kinase directly phosphorylates Mid1. Phosphosite mapping and phenotypic analyses of phosphosite mutants reveal defects in Mid1 nuclear accumulation following septation and during interphase. Instead, Mid1 remains concentrated in the cytoplasm and appears to localize as cortical nodes. Strikingly, a reproducible percentage of phosphosite mutants display two contractile rings and/or disorganized contractile rings. To further examine Mid1 localization, we combined the Mid1 phosphosite mutant with a temperature-sensitive mutation that arrests cells at anaphase when Mid1 should be concentrated in the nucleus. In the phosphosite mutant, Mid1 fails to move from the cytoplasm to the nucleus. Instead Mid1 accumulates in punctate clusters or remains in rings that are often abnormal. We conclude that Sid2 phosphoregulation of Mid1 nuclear accumulation during interphase promotes proper contractile ring function in mitosis.

This research is supported by National Science Foundation RUI Award #1157997.

P882**Board Number: B201****CARMIL proteins exhibit isoform-specific spatial and temporal expression patterns during zebrafish development.**B.C. Stark¹, J.A. Cooper¹;¹Department of Cell Biology and Physiology, Washington University School of Medicine, Saint Louis, MO

Actin assembly is essential for many cellular processes, including cell migration during development, an immune response, and metastasis. Assembly occurs primarily at barbed ends of actin filaments, and the generation of new barbed ends is highly regulated. Capping protein binds to actin barbed ends and stabilizes the filament, preventing loss or addition of actin subunits. CARMIL proteins are a major regulator of capping protein. They are found throughout vertebrates and in some lower eukaryotes including *Acanthamoeba* and *Dictyostelium*. CARMILs bind to and inhibit capping protein via an

allosteric mechanism. Most exciting, they are able to remove capping protein from barbed-ends and thereby generate a new, free barbed-end. CARMILs are large, multi-domain proteins. Vertebrate genomes contain three conserved genes that encode three CARMIL isoforms. Prior to my work, only one CARMIL isoform had been confirmed in zebrafish. I have identified two additional CARMIL genes in the zebrafish genome. All three isoforms of CARMIL in zebrafish are highly conserved with respect to other vertebrates. All three are expressed during development. The CARMIL 1 and CARMIL 3 isoforms are expressed both maternally and zygotically, whereas the CARMIL 2 isoform is expressed zygotically. RNA in situ hybridizations reveals differential expression patterns for all three genes within the developing embryo. Morpholino-mediated knockdowns of CARMIL 2 and CARMIL 3 result in isoform-specific phenotypes. CARMIL 2 knockdown results in embryonic lethality in 90% of fish by 24 hpf. CARMIL 3 knockdown results in cardiac defects and death by 5 dpf.

P883

Board Number: B202

A temporally ordered mechanism for actin filament disassembly involving mammalian Coronin, Cofilin, AIP and Srv2/CAP.

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The actin cytoskeleton is a self-organizing, dynamic polymer system that drives important biological processes such as endocytosis, cell migration and morphogenesis, and relies on balanced actin filament assembly and disassembly. How actin disassembly is regulated is still not well understood. One of the most well characterized components of the disassembly machinery is Cofilin/ADF, which binds cooperatively to filaments and induces severing. However, Cofilin binds F-actin with surprisingly slow kinetics, has relatively inefficient severing activity, and produces free (uncapped) barbed ends, which leads to assembly instead of disassembly. These enigmas have long suggested that additional cellular factors may be required in vivo for rapid and efficient F-actin disassembly. Here, we used three-color TIRF microscopy single molecule analysis to investigate the mechanism by which mammalian Coronin, AIP1 and Srv2/CAP (cyclase-associated protein) work with Cofilin to promote actin disassembly. We show that these four proteins work in a specific order on ADP-F-actin and together produce ultra-fast disassembly. Coronin binds first to filaments and rapidly recruits Cofilin. Then, AIP1 arrives and induces highly efficient severing. After severing, AIP1 remains associated with the newly generated barbed ends, blocking growth and promoting disassembly even under assembly conditions. Srv2/CAP makes a distinct mechanistic contribution to disassembly by binding autonomously to filament sides and altering F-actin structure to enhance Cofilin-dependent severing. Finally, in a reconstituted assembly-disassembly system, we could directly visualize formins rapidly polymerizing filaments, counterbalanced by equally rapid severing and depolymerization by the disassembly factors working in concert.

P884**Board Number: B203****Tropomodulin4-deficient mice exhibit compensatory upregulation of tropomodulin1 and altered thin filament activation in skeletal muscle.**D.S. Gokhin¹, J. Ochala², A.A. Domenighetti³, V.M. Fowler¹;¹Department of Cell and Molecular Biology, The Scripps Research Institute, La Jolla, CA, ²Centre of Human and Aerospace Physiological Sciences, King's College of London, London, United Kingdom,³Department of Orthopaedic Surgery, University of California, San Diego, La Jolla, CA

Simultaneous control of thin filament lengths, thin filament activation, and actomyosin crossbridge formation is essential for efficient contractile function of skeletal muscle fibers. Thin filament pointed ends in skeletal muscle sarcomeres are capped by a combination of two sarcomeric tropomodulin (Tmod) isoforms, Tmod1 and Tmod4, both of which interact with the N-terminus of tropomyosin and regulate actin subunit dynamics *in vitro*. However, to date, only Tmod1 has been directly implicated in the specification of muscle-specific thin filament lengths, formation of actomyosin crossbridges, and contractile stress generation *in vivo*. To better understand the *in vivo* functions of Tmod4 and distinguish Tmod4's functions from Tmod1's functions, we first compared Tmod1 vs. Tmod4 protein levels across various muscle types using quantitative western blotting. The ratio of Tmod4:Tmod1 protein levels ranged from ~9.5:1 in a representative fast muscle (tibialis anterior) to ~4:1 in a representative slow muscle (soleus), suggesting that sarcomeric Tmod isoform distributions are muscle-specific, analogous to tropomyosin, troponin, and myosin isoform distributions. This is partly due to muscle-specific differences in gene expression, as RNA sequencing analysis identified ~2-fold greater *Tmod4* mRNA levels in tibialis anterior muscle than in soleus muscle, but markedly lower *Tmod1* mRNA levels in both of these muscle types. Next, we investigated the functional role of Tmod4 *in vivo* by characterizing the skeletal muscle phenotype of a novel *Tmod4*^{-/-} mouse. *Tmod4*^{-/-} mice exhibit no overt myopathic features, with normal myofibril organization and unchanged thin filament lengths, due to compensatory upregulation of Tmod1 of a magnitude sufficient to repopulate nearly all of the pointed ends vacated as a result of Tmod4 deletion. Thus, Tmod4 is dispensable for specification of thin filament lengths *in vivo*. However, the switch from pointed-end capping by Tmod1 and Tmod4 in wild-type muscle to exclusively Tmod1 in *Tmod4*^{-/-} muscle resulted in a trend toward reduced and more variable Ca²⁺ sensitivity during thin filament activation, although the kinetics of actomyosin crossbridge cycling, the total number of myosin heads bound, and maximal isometric stress generation were all unchanged. We propose that muscle-specific isoform distributions of Tmod1 and Tmod4 may enable muscle-specific, collaborative fine-tuning of thin filament lengths and thin filament activation, possibly mediated by long-range effects initiated by Tmod-tropomyosin interactions at the pointed end.

P885**Board Number: B204****Comparison of actin-binding activity between MyBP-C (myosin-binding protein-C) isoforms.**S. Wakatsuki¹, T. Obinata¹, N. Sato¹;¹Department of Nanobiology, Chiba University, Graduate school of Advanced Integration Science, Chiba, Japan

Myosin-binding protein-C (MyBP-C/ C-protein) is one of the major myosin-binding proteins and has three isoforms, cardiac (cMyBP-C), fast-skeletal (fMyBP-C) and slow-skeletal type (sMyBP-C) in vertebrate striated muscles. MyBP-C locates at the C-zone of A-band in sarcomeres of vertebrate striated muscles. The fMyBP-C and sMyBP-C are composed of 3 fibronectin type III (FNIII) repeats and 7 immunoglobulin (Ig) domains. The cMyBP-C has an extra Ig domain (C0) at the N-terminus. A linker sequence between domain 1 and 2 is named MyBP-C motif and contains unique phosphorylation sites in the cardiac isoform, which imply additional functions compared with the skeletal isoforms; e.g., mutation of human cMyBP-C causes to hypertrophic cardiomyopathy. It has been demonstrated that the N-terminal domains of cMyBP-C interact with actin filaments and S2 subfragment of myosin, on the other hand, the C-terminal domains interacts with light meromyosin and connectin/titin. Through these domains, cMyBP-C modulates myosin assembly, actin-myosin interaction, and stabilizes thick filaments. However, it is unclear whether fMyBP-C and sMyBP-C bind to actin filaments and affect actomyosin interaction. In this study, we prepared N-terminal fragment encoding mouse cMyBP-C (domain 0 to domain 3), that of fMyBP-C (domain 1 to domain 4) and that of sMyBP-C (domain 1 to domain 4) in an *E. coli* expression system. Cosedimentation assays and other experiments revealed that not only cMyBP-C but also fMyBP-C and sMyBP-C bind to actin filaments by a cross-linking (or bundling) manner, and also bind to myosin filaments at the N-terminal regions. When the fMyBP-C fragment and actin were incubated in a molar ratio of 0.25:1, the fMyBP-C elevated actin-activated myosin ATPase activity significantly. To determine the actin filaments and myosin filaments binding site of fMyBP-C precisely, we prepared several deletion fragments of fMyBP-C. The result indicated that fMyBP-C interacts with actin filaments and myosin filaments through its MyBP-C motif.

P886**Board Number: B205****Arginylation of beta actin is dynamically regulated during cell migration.**D. Volgin¹, A.S. Kashina¹;¹Department of Animal Biology School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA

Our previous work has uncovered that beta actin is arginylated in non-muscle cells and that this arginylation is required for leading edge formation during cell migration, but the underlying mechanisms are not understood. Here we used a newly developed antibody to arginylated beta actin

and found that arginylation dynamically increases in cells that are actively migrating and reduces during resting period, and that arginylated beta actin is concentrated in the leading lamella in a zone located right behind the leading edge actin network. Lack of arginylation results in a dramatic reduction of actin polymer levels in the lamellipodia. Our data suggest that arginylation of beta actin is a tightly regulated process that occurs in response to cell migration stimuli and is required for the massive polarized growth of the actin filaments that facilitates leading edge protrusion.

P887

Board Number: B206

Arginylation regulates brain development, neuronal patterning, and growth cones.

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Arginylation is an emerging posttranslational modification that has been recently found to affect actin cytoskeleton and regulate cell migration in non-muscle cells. Here we studied the effect of the brain-specific knockout of arginyltransferase (Ate1) on mouse development and the morphology and behavior of neurons in a conditional knockout model with Ate1 deletion driven by brain-specific Nestin (Nes) promoter. Such Nes-Ate1 mice exhibit high rates of perinatal lethality accompanied by prominent structural and behavioral retardation. Cultured Ate1 knockout neurons isolated from embryos and neonates exhibit delayed growth cone development. Immunofluorescence studies show that arginylated beta actin prominently accumulates in developing wild type neurons and appears to target specifically to the growth cones during branching, following only the protruding neurites. Our results suggest that Ate1-mediated arginylation of beta actin is actively involved in neuron development and outgrowth and that its abolishment leads to several neuronal impairments at cellular and organismal level.

P888

Board Number: B207

Regulation of pheromone-induced cell polarization by the polarisome proteins in yeast.

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The ability to polarize is essential for the function of many cell types. During mating, haploid budding yeast cells polarize growth to generate mating projections (shmoo) towards each other in order to fuse and form diploids. Such pheromone-induced cell polarization and morphogenesis is thought to be regulated by the “polarisome” proteins Bni1, Bud6, Pea2, and Spa2. These proteins are thought to act

primarily by localizing formin activity, which acts to orient actin cables that deliver secretory vesicles to the polarity site. In turn, polarisome components are themselves targeted to the polarity site by the master polarity regulators, Cdc42 and Bem1. Here we report interdependencies between Spa2, Pea2, and Bni1 for localization to the polarity site. In addition, we find that loss of Spa2 or Pea2 may alter the distribution of upstream regulators, as well as weakening downstream vesicle targeting. Vesicle accumulation at the shmoo tip was most severely reduced in cells lacking the formin Bni1. However, we found that loss of all formins did not block polar growth or the formation of mating projections, suggesting that secretory vesicles can be directed to the polarity site independent of actin cables.

P889

Board Number: B208

CD44/Integrin α v β 3 signaling: Role in Prostate Cancer Invasion/Metastasis and Osteoclastogenesis.

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Pathological skeletal fractures develop in prostate cancer patients as a result of bone loss induced by androgen deprivation therapy and bone metastases. In this regard, bone-resorbing osteoclasts (OCs) are essential in mediating bone loss induced by metastatic prostate cancer cells homing to the skeleton. Therefore, there is a critical need for novel pharmacotherapeutic approaches to control this debilitating disease. We have previously demonstrated that prostate cancer cells secrete receptor activator of NF- κ B ligand (RANKL), a protein essential for OC differentiation and activation. The objective of our study is to inhibit the expression and function of RANKL in metastatic prostate cancer cells. We used prostate cancer cells lines derived from a variety of anatomic metastases. We found a significant increase in the expression of CD44, a cell surface receptor in prostate cancer cells derived from human bone metastases (PC3) as compared with those derived from brain (DU 145) or lymph node (LNCaP) metastases. CD44 expression is very negligible or not observed in LNCaP cells. We show here that CD44 / Src signaling which involves cortactin-WASP-Arp2/3 axis plays an important role in the formation of invadopodia and invasion of PC3 cells. Knock down of CD44 and expression of kinase defective Src in PC3 cells reduces invasive property of PC3 cells. RUNX2 and Smad 5 proteins are involved in the regulation of expression of RANKL. CD44 and α v β 3 signaling pathways support RANKL expression by phosphorylation of RUNX2 and Smad 5 proteins, respectively. RUNX2-Smad 5 complex formation and intranuclear targeting of RUNX2 are functionally required for this process. An inhibitor to integrin α v and SiRNA to CD44 attenuated the expression of RANKL in PC3 cells. Immunohistochemistry analysis of tissue microarray sections containing primary prostatic tumor (grade2-4) detected predominant localization of RUNX2 and phosphorylated Smad 5 in the nuclei. Small molecular weight CD44 peptides have the potential to block RANKL expression/secretion and conditioned media from these cells blocked OC formation in vitro although at different levels. These results provide an experimental basis for the development of anti-invasive/metastasis peptide agent to block pathways possibly involved in invadopodia formation and osteoclastogenesis.

P890**Board Number: B209****Spatiotemporal mathematical modeling of myocardin-related transcription factor-A signaling.**B. Spar¹, Q.K. Chen², C. Nelson^{2,3};¹Computer Science, Princeton University, Princeton, NJ, ²Chemical and Biological Engineering, Princeton University, Princeton, NJ, ³Dept. of Molecular Biology, Princeton University, Princeton, NJ

Myocardin-related transcription factor-A (MRTF-A; also known as Mkl1, MAL, BSAC) is involved in many cellular processes including migration, organogenesis and tumor metastasis. MRTF-A is activated by nuclear translocation, which is regulated by the nucleocytoplasmic distribution of monomeric and filamentous actin. The current conceptual model is that MRTF-A is only exported from the nucleus when bound to G-actin, and only imported when unbound. To test the robustness of this conceptual model, we transfected MRTF-A under a strong constitutive promoter into NIH-3T3 fibroblasts. MRTF-A nuclear localization was quantified using immunostaining.

Surprisingly, we found that the nuclear localization of MRTF-A was altered in cells expressing ectopic protein compared to endogenous. Specifically, treatment with cytoskeletal disruptors such as jasplakinolide or cytochalasin D, or stimulation with serum, had different effects on localization of ectopic versus endogenous MRTF-A. Computational kinetic modeling of the MRTF-A/actin regulatory axis using systems of differential equations was unable to quantitatively explain these results. Stochastic kinetic modeling using the slow-scale stochastic simulation algorithm, a variant of the Gillespie algorithm, was unable to reproduce this distribution using physiologically relevant protein copy numbers. Taken together, our results show that the currently accepted conceptual model of MRTF-A nucleocytoplasmic shuttling does not sufficiently explain MRTF-A kinetics.

P891**Board Number: B210****Multilevel crosstalk between Myocardin-related transcription factor (MRTF) and TAZ, two major cytoskeleton-regulated transcriptional coactivators: role in myofibroblast transition.**P. Speight¹, A. Kapus²;¹Keenan Research Center for Biomedical Science, Toronto, ON, ²University of Toronto, Depts Surgery, Biochemistry, Keenan Research Center for Biomedical Science, Toronto, ON

The cytoskeleton impacts gene transcription and thus cell plasticity by regulating the nucleocytoplasmic traffic of mechanosensitive transcription factors. Of these, the Rho effector MRTF and the Hippo pathway target TAZ have emerged as key molecules linking actin dynamics and gene expression. While the nucleocytoplasmic shuttling of MRTF is regulated by its direct binding to G-actin, the mechanism

whereby the cytoskeleton controls TAZ remains unknown. Since we noticed that MRTF contains a C-terminal WW domain-binding motif, while TAZ has a WW domain, we asked if MRTF and TAZ might associate and if MRTF could confer actin-sensitivity to TAZ via a piggyback mechanism. Here we show that MRTF and TAZ indeed bind each other but MRTF is not responsible for the actin-dependent nuclear uptake of TAZ. However, MRTF and TAZ do impact each other at multiple levels, influencing (either unilaterally or reciprocally) the expression, nuclear traffic, mobility and transcriptional activity of the partner. Coimmunoprecipitation studies revealed that MRTF and TAZ associate both in the cytoplasm and in the nucleus. Mutation of the WW-binding motif caused 60% reduction in TAZ binding to full-length MRTF and abolished binding to the isolated C-terminus. MRTF silencing drastically reduced TAZ mRNA and protein expression, indicating that MRTF is a key transcription factor for TAZ. Knockdown of TAZ did not affect MRTF expression, but doubled MRTF's cytosolic mobility (as measured by FRAP) and strongly facilitated its stimulus-induced nuclear accumulation. Cytosolic retention of MRTF by TAZ required the WW-binding motif. Conversely, silencing of MRTF enhanced the nuclear accumulation of heterologously expressed TAZ. Since our previous studies have shown that both MRTF and TAZ impact the expression of the myofibroblast marker α -smooth muscle actin (SMA), we examined their transcriptional effects on the SMA promoter, which harbors side-by-side cis-elements for MRTF (CArG boxes) and TAZ (Tead-binding elements, TBE). MRTF (strongly) and TAZ (weakly) stimulated the SMA promoter through CArG and TBE, respectively, while they reciprocally inhibited each other's effect. TAZ suppressed the MRTF-induced activation of the wild type promoter, while inactivation of the CArG boxes strongly augmented the effect of TAZ. In contrast, TAZ conferred sensitivity for the TGF β effector, Smad3 through Smad-binding elements. Based on these findings, we propose a model in which MRTF and TAZ/Smad3 represent two alternative modes of regulation of SMA expression, which may be linked to wound healing-associated (MRTF) vs. fibrogenic (TAZ/Smad3) programming of myofibroblasts. Selective targeting of these pathways offers a new strategy to interfere with SMA expression only in aggressive myofibroblasts.

P892

Board Number: B211

Dystroglycan depletion inhibits the functions of differentiated Kasumi-1 cells.

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Hematopoiesis is a complex process, requiring self-renewing and pluripotent stem cells, differentiated progenitor and precursor cells, as well as supportive stroma, adipose tissue, vascular structures, and extracellular matrix. The extracellular matrix guides their division, growth, and development, determining functions. The dystroglycan comprises two glycoproteins; the N-terminal peripheral membrane protein α -dystroglycan that links to the extracellular matrix via several ligands, whereas the

transmembrane β -dystroglycan links α -dystroglycan to the actin cytoskeleton via dystrophin or utrophin. Dystroglycan is widely expressed and heavily glycosylated, the glycosylation pattern of dystroglycan varies with development and tissue and is essential for its function. The central role of dystroglycan as a link between cytoskeleton and extracellular matrix clearly defines dystroglycan as an adhesion molecule. Dystroglycan has recently been characterized in blood tissue cells, as part of the dystrophin glycoprotein complex but to date nothing is known of its role in the induction of macrophagelike cells. We have characterized the pattern expression and cellular distribution of dystroglycans in Kasumi-1 cell line, a human acute myeloid leukaemia cell line differentiated to macrophagelike cells and we have investigated the role of dystroglycan during the differentiation process. Depletion of dystroglycan by RNAi resulted in altered morphology and reduced properties of differentiated Kasumi-1 cells, including chemotaxis, phagocytic activities and expression of markers of differentiation. These findings strongly implicate dystroglycan as a key membrane adhesion protein involved in the differentiation process in Kasumi-1 cells and so is therefore of considerable functional importance.

P893

Board Number: B212

MIEN1 drives breast cancer invasion by regulating cytoskeletal dynamics.

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Introduction: The emergence of metastases remains the primary cause of cancer mortality and morbidity. Active migration of tumor cells is a prerequisite for tumor-cell invasion and metastasis. We identified Migration and Invasion Enhancer 1(MIEN1) as a novel promoter of cell migration/invasion. Previously known as C35, C17orf37, RDX12 and MGC14832, MIEN1 is a novel gene located in the chromosomal region 17q12-21. While absent or low in normal tissues, MIEN1 is abundantly expressed in multiple cancers; including breast, prostate, oral and gastrointestinal carcinomas. A membrane-bound signaling adaptor, MIEN1 localizes to the leading edge of migrating cells and promotes migration and invasion by increasing filopodium formation. While MIEN1 is shown to promote motility through filopodia formation, little is known on the mechanisms by which MIEN1 influence the actin cytoskeleton. Exploring underlying mechanisms, we show that MIEN1 associates with the actin cytoskeleton during motility and directly influence actin polymerization. **Methods:** We performed immunofluorescence, migration and invasion assays using breast cancer cell lines, MDA-MB 231 and MCF10CA1a to investigate the mechanisms by MIEN1 potentiate cell motility.

Results: Ablation of MIEN1 inhibited cell migration, and altered the expression of downstream targets. Immunofluorescence staining with FITC- conjugated-phalloidin confirmed that MIEN1 induced-migration is associated with actin filaments and the knockdown of MIEN1 disturbs the actin cytoskeleton in both MDA-MB231 and MCF10CA1a breast cancer cells. Examination of F-actin and G-actin contents in MIEN1 knocked-down cells showed that the mean ratio of F-actin to G-actin was significantly lesser than control cells. **Conclusion:** Our results show that MIEN1 is a critical regulator of cell motility. The importance of MIEN1 in tumor cell dissemination is well established; understanding the

molecular mechanisms aiding the processes involved will enable designing novel and effective treatments for metastatic tumors

P894

Board Number: B213

Role of Grb2 in muscle differentiation.

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Grb2 (Growth Factor Receptor bound protein-2) is an adaptor protein which is known to interact with tyrosine phosphorylated- Epidermal Growth Factor Receptor (EGFR) through its SH2 domain and N-WASP through its SH3 domain. N-WASP belongs to the Wiskott Aldrich Syndrome Protein (WASP) family which regulates actin cytoskeleton remodeling through activation of Arp2/3 complex. Reorganization of actin cytoskeleton is critical for various processes such as muscle differentiation, endocytosis, cancer cell migration and invasion, etc. N-WASP has been shown to be required for the process of muscle differentiation, which involves step wise fusion of mono-nucleated myoblasts to form multi-nucleated myotubes. However the role of Grb2 in myogenesis has not been elucidated. In order to study the role of Grb2 in myogenesis we generated a Grb2 knocked down C2C12 cell line (Mouse myoblast) and found that knocking down Grb2 enhanced myogenic differentiation even though the expression of Grb2 was found to be relatively unchanged during myogenic differentiation. This suggests that Grb2 might have an inhibitory role in myogenic differentiation. We are currently generating Grb2 overexpressing C2C12 cells to characterize the inhibitory role of Grb2 in myogenesis. Identifying the role of Grb2 in muscle differentiation will give us a better understanding about the role of N-WASP-Grb2 complex in repair and ageing.

P895

Board Number: B214

USP7 inhibitors for the treatment of malignancies.

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Ubiquitylation and deubiquitylation control the stability, localization and activity of many cellular proteins. Deubiquitylation is performed by a family of ~80 enzymes (DUBs) including USP7, which removes ubiquitin selectively from HDM2, Foxp3, claspin and various other therapeutically relevant substrates. Importantly, USP7 is overexpressed in prostate cancer and human multiple myelomas and the expression level of USP7 is inversely correlated with prognosis in multiple myeloma patients. Previously, we reported the discovery and characterization of P0005091 a USP7 inhibitor which exhibited anti-tumor activity in in vitro and in vivo models of multiple myeloma. Additionally, we

reported that P0005091 inhibits USP47, a closely related DUB that is an emerging oncology target in its own right. Furthermore, a structural analog of P0005091, P0022077 has recently been reported by another research group to inhibit neuroblastoma growth in an orthotopic mouse model, suggesting that in addition to hematological tumors, solid tumors may be treatable via inhibition of USP7. To obtain additional starting points for a USP7 or a USP7/USP47 selective drug discovery program we recently conducted a new screening campaign. By utilizing a biophysical assay platform we were able to exclude compounds that bind to the active site of USP7, thereby increasing the possibility of identifying allosteric modifiers of USP7 activity. Hits from this screen are currently the subject of hit to lead optimization and will be evaluated in biophysical and biochemical assays as well as in vitro and in vivo models of hematological and solid tumors. Data will be presented describing these results.

P896

Board Number: B215

Dexamethasone And Azathioprine Promote Cytoskeleton Changes And Affect MSCs Migratory Behavior.

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Glucocorticoids and immunosuppressive drugs are commonly used to treat inflammatory disorders, such as Inflammatory Bowel Disease (IBD) and, despite few improvements, the remission of IBD is still difficult to maintain. Due to its immunomodulation properties, Mesenchymal Stem Cells (MSCs) have emerged as regulators of immune response, and its viability and activation of migratory properties are essential for successful cell therapy. However, little is known about the effects of immunosuppressant drugs used on IBD treatment on MSCs behavior. The aim of this study was to evaluate MSC viability, nuclear morphometry, cell polarity, F-actin distribution and cell migration properties in the presence of the immunosuppressive drugs Azathioprine (AZA) or Dexamethasone (DEX). After initial characterization, MSCs were treated with DEX (10 μ M) or AZA (1 μ M) for 24 hrs or 7 days. Both drugs had no effects on cell viability or nuclear morphometry. However, AZA treatment induced a more elongated cell shape and increased the presence of ventral actin stress fibers, while DEX was associated to a more rounded cell shape with high presence of ventral actin stress fibers and a decrease on protrusion stability. After 7 days of treatment, AZA improved cell the spatial trajectory (ST) and increased migration speed (24.35%, $P < 0.05$, $n = 4$) while DEX impaired ST and migration speed after 24 hrs and 7 days treatment (-28.69% and -25.37%, respectively; $P < 0.05$, $n = 4$). In conclusion our data suggests these immunosuppressive drugs can differently affect MSCs morphology and migration capacity, possibly impacting the success of cell therapy.

Keywords: Mesenchymal Stem Cells, Actin, Cell Migration, Azathioprine, Dexamethasone, Inflammatory Bowel Disease.

P897

Board Number: B216

14-3-3 β , but not 14-3-3 γ , Supports Nuclear Transport of Synaptopodin 2 (Myopodin).

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Multiple actin binding proteins shuttle between the nucleus, the cytoplasm, or the membrane, depending on environmental and cell differentiation parameters. Synaptopodin 2 (SYNPO2 in the sequel) is one of these proteins. Via expression of ECFP or EYFP tagged proteins, as well as with a fluorescence complementation assay (BIFC), we were able to confirm the validity of earlier in vitro observations within the intact cellular environment. Rat synaptopodin 2 (syn. myopodin), importin α , and 14-3-3 β/γ , were cloned into conventional pECFP and pEYFP vectors, as well as into complementary split-EYFP vectors. In HEK293 cells transfected with the conventional vectors, confocal microscopy localized EYFP-SYNPO2 to the cytoplasm and to the nucleus, with accumulation in the nucleoli. Importin α and 14-3-3 β were also found in the nucleus, however, this was not the case for 14-3-3 γ . The BIFC-assay revealed that SYNPO 2 formed cytoplasmically located dimers and also interacted with importin α and 14-3-3 β/γ . In the nucleus, we did not observe SYNPO 2 dimers or an interaction of SYNPO 2 with 14-3-3 γ . However, SYNPO 2 formed stable intra-nuclear complexes with importin α and 14-3-3 β , which participated in loop-formation and had the appearance of actin-loops. While in vitro studies and our cellular BIFC-assays demonstrated that 14-3-3 β supports nuclear translocation of SYNPO 2, we did neither detect 14-3-3 γ -SYNPO 2 complexes in the nucleus nor did we find intra-nuclear 14-3-3 γ complexes. This is surprising since 14-3-3 γ , at least in smooth muscle cells, is abundant and we expected that it may substitute there for the less abundant 14-3-3 β . Thus, while SYNPO 2 is able to interact with both isoforms of 14-3-3 β and γ , in the cytosol, only the β -isoform appears to trigger nuclear translocation of SYNPO 2.

P898**Board Number: B217****Comparison Of Incorporation of Wild Type And Mutated Actins Into Sarcomeres in Skeletal Muscle Cells: A Fluorescence Recovery After Photobleaching (FRAP) study.**J. Wang¹, Y. Wang¹, J.M. Sanger¹, J.W. Sanger¹;¹Cell and Developmental Biology, SUNY Upstate Med Univ, Syracuse, NY

Our long-term goals are to understand how the highly ordered arrangements of myofibrils form and how this complex of interacting proteins is maintained over a lifetime of contractile activity. Failure to achieve optimal assembly and function can be caused by many factors, among the most serious of which are mutations in myofibrillar proteins that cause severe myopathies. Analyzing such mutated proteins in live muscle cells undergoing myofibrillogenesis should yield insights into the molecular effects of these mutated proteins. The actin mutation, G15R, is located in the nucleotide-binding pocket and causes a severe actin myopathy in human skeletal muscle cells. When expressed in cultured skeletal muscle cells, G15R is incorporated into the sarcomeres in a pattern initially indistinguishable from wild type actin. However, patches of mutant actin resembling patches found in patient biopsies subsequently form. FRAP analysis of incorporation of mutant actin localized in myotubes shows major differences from incorporation of wild type sarcomeric actin when the data are plotted not as average FRAP recovery, but as a function of the fast-growing ends of the actin filament in the Z-band, versus the slow growing ends of the thin filaments in the middle of the sarcomeres. In cultured skeletal muscle, wild type actin shows a markedly faster rate of incorporation at the plus (+) ends of actin filaments than at the minus (-) ends of the filaments. By comparison with wild type actin, the incorporation of mutated actin-G15R molecules is reduced at the plus (+) ends and increased at the minus (-) ends. The same relationship of incorporation is seen in cytochalasin-D inhibited myofibrils: decreased dynamics at the plus (+) ends and increased dynamics at minus (-) ends of actin filaments. We speculate that the imbalance of the normal polarized assembly of actin filaments creates excess monomers that form actin patches. Our results indicate that normal actin plus (+) end dynamics are needed to maintain actin thin filament stability in the myofibrils.

P899**Board Number: B218****Biochemical and Biological Properties of Dictyostelium Cortexillin III and Its Complex(es) with DGAP1.**S. Shu¹, X. Liu², S. Yu², D. . Lee³, G. Wang², M. Gucek², E.D. Korn²;¹NHLBI, National Institutes of Health, Bethesda, MD, ²NHLBI, NIH, Bethesda, MD, ³NIH/NHLBI, Bethesda, MD

Cortexillins I, II and III are members of the α -actinin/spectrin sub-family of Dictyostelium calponin homology proteins. We report the biochemical properties of recombinant cortexillin III, the complexes formed by FLAG- and GFP-tagged cortexillin III expressed in Dictyostelium, and the properties of cortexillin III-null cells. Purified recombinant cortexillin III is an unstable monomer, unlike recombinant cortexillins I and II which form stable homodimers in vitro. However, cortexillin III forms more stable heterodimers when co-expressed in E. coli with either cortexillin I or cortexillin II, and co-expressed cortexillin I and cortexillin II also form stable heterodimers. All of the recombinant homodimers and heterodimers bind weakly to F-actin and none affects actin polymerization in vitro. As determined by both co-immunoprecipitation, and by affinity chromatography and gel filtration, cortexillin III expressed in Dictyostelium heterodimerizes with both cortexillin I and II. The heterodimers form one or more complexes with DGAP1, a Dictyostelium GAP protein. Like cortexillins I and II, GFP-cortexillin III expressed in wild-type Dictyostelium localizes with F-actin in the cortex of vegetative cells, the leading edge of motile cells, and the cleavage furrow of dividing cells. However, although expressed cortexillin III localizes with F-actin in both cortexillin I and cortexillin II single-null cells, cortexillin III is diffuse in the cytoplasm when expressed in cortexillin I/ II double-null cells and also when expressed in DGAP1-null cells. We conclude from these data that cortexillin III localizes with F-actin in vivo only as a heterodimer with either cortexillin I or cortexillin II complexed to DGAP1-complex. As neither homodimers nor heterodimers of recombinant cortexillin I and cortexillin II bind with high affinity to F-actin in vitro, we speculate that localization of cortexillin I and cortexillin II with F-actin in vivo may occur only when complexed to DGAP1 or GAPA (a Dictyostelium GAP protein to which cortexillins I and II, but not cortexillin III, also bind).

Deletion of cortexillin III affects chemotaxis and development. Although individual cortexillin III-null cells chemotax normally in a cAMP gradient they form unstable streams, and starved cortexillin III-null cells form much smaller mounds and fruiting bodies than wild-type cells. Also, cortexillin III-DGPA1 complexes may be negative regulator of pinocytosis, phagocytosis, cytokinesis and cell growth as all are enhanced in cortexillin III-null cells.

P900**Board Number: B219****MARVELD1 Alters Malignant Phenotype of Lung Cancer Cell via Modulating Cell Adhesion Signaling.**Y. Yao¹;¹School of Life Science and Technology, Harbin Institute of Technology, Harbin, China

MARVELD1 (MARVEL domain-containing 1) is a member of MARVEL (MAL and related proteins for vesicle trafficking and membrane link) family. Recently, we reported MARVELD1 inhibited cell migration and regulated cell adhesion in epithelial cells. However, how MARVELD1 functions in lung tumorigenesis is still not understood. We now demonstrate that low MARVELD1 expressing lung cancer cells have significant defects in cell spreading as well as increased cell metastasis. The MARVELD1-mediated Integrin β signaling activated downstream adhesion molecules FAK, Talin and Paxillin, and suppressed matrix metalloproteinases MMP2 and MMP3. In addition, we also found MARVELD1-mediated Integrin signaling regulated filopodial length and density of lung cancer cells. The numerous filopodia that induced by MARVELD1 expression may be a prerequisite for the cells to form adherens junctions. Scanning electron microscope (SEM) analysis further determined that MARVELD1 expression reduced the aggressive character of lung cancer cells. Collectively, our data showed MARVELD1-mediated Integrin signaling alters the malignant phenotype of lung cancer cells, suggesting MARVELD1 has a potential to be developed as a therapeutic target for lung carcinoma.

P901**Board Number: B220****Arp2/3 Complex Inhibition Radically Alters Lamellipodial Actin Architecture, Suspended Cell Shape, and the Cell Spreading Process.**J.H. Henson¹, H. Geist², M. Pais²;¹Dickinson Coll, Carlisle, PA, ²Dickinson College, Carlisle, PA

Recent studies have investigated the dendritic actin cytoskeleton of the cell edge's lamellipodial (LP) region by experimentally decreasing the activity of the actin filament nucleator and branch former the Arp2/3 complex. Here we extend these studies via pharmacological inhibition of the Arp2/3 complex in sea urchin coelomocytes, cells that possess an usually broad LP region and display correspondingly exaggerated centripetal flow. Using light and electron microscopy, we demonstrate that Arp2/3 complex inhibition via the drug CK666 dramatically altered LP actin architecture, slowed centripetal flow, drove a lamellipodial to filopodial shape change in suspended cells, and induced a novel actin structural organization during cell spreading. A general feature of the CK666 phenotype in coelomocytes was transverse actin arcs and arc generation was arrested by a formin inhibitor. We also demonstrate that CK666 treatment produces actin arcs in other cells with broad LP regions, namely fish keratocytes and *Drosophila* S2 cells. We hypothesize that the actin arcs made visible by Arp2/3 complex inhibition in

coelomocytes represent an integral feature of the LP cytoskeleton and may correspond to the elongate mother filaments that serve as the scaffold for the production of the dendritic actin network.

P902

Board Number: B221

14-3-3 controls cell cortical tension in human cells.

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The cell cortex is mainly composed with a thin meshwork of actin filaments, myosin and associated proteins that lie beneath the plasma membrane. The tension is created by the active myosin motors when they slide actin filaments with respect to one another in the network. The tension is regulated by the myosin motor activity and the stiffness of actin network which is regulated by other associated proteins. At cellular level, the tension of cell cortex is tightly regulated spatially and temporally during cell shape change of any cellular process, such as cytokinesis and directional migration. At tissue level, the cell cortical tension may also underlie part of the tissue surface tension, which is one of the most direct biomarker for physicians to differentiate tumor tissue from normal surrounding tissues. In our previous study, we found that 14-3-3 regulates cell cortical tension in *Dictyostelium* cells, where 14-3-3 down-regulation leads to decrease of cortical tension and cytokinesis failure. In this study, we investigated 14-3-3's role in cortical tension regulation in mammalian cells. Our preliminary data indicates that when 14-3-3 sigma is knocked down by siRNA in Hela S3 cells, cell cortex becomes much softer when comparing with control cells. In the near future, cell transwell migration assay will be used to study how 14-3-3 may impact cell migration capability and survival rate of cells growing with shear stress will be assessed when 14-3-3 is down-regulated as well. We hypothesize that down-regulation of 14-3-3 leads to decrease of cell cortical tension, which may increase the migration capability and yet reduce the survival rate when cells are stressed with shear stress. Our study may suggest 14-3-3's complicated roles in cancer cell metastasis.

Regulation of Actin Dynamics 2

P903

Board Number: B222

The formin INF2 is a focal adhesion protein that promotes dorsal stress fiber and fibrillar adhesion formation to drive extracellular matrix assembly.

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The dynamic connection between actin and focal adhesions (FA) is essential for driving cell migration and tissue morphogenesis. Actin and FA frequently behave as an integrated system, and exhibit remarkable plasticity in form, with actin establishing dendritic meshworks and contractile and non-contractile bundles, and FA maturing from tiny nascent adhesions, to focal complexes, mature FA, and fibrillar FA. Some actin/FA systems are well understood, for example, Arp2/3 mediating the formation of a lamellipodium containing nascent FA that is required for cell guidance by haptotaxis. However, motile cells display a number of other actin-based structures, such as contractile arcs and dorsal stress fibers in the lamella, TAN lines over the nucleus, and ventral stress fibers under the cell body, yet the mechanism of their formation and how they interface with FA to specify function is not known. The formin family of actin nucleators is thought to be critical for formation of many linear actin structures, including dorsal SF at FA, however none of these family members have been found to localize to FA. We previously identified the formin INF2 in a proteomic screen of FA proteins, and thus sought to analyze its role in FA and SF in mouse embryonic fibroblasts. We found that INF2 localized to FA in the lamella at their junction with SF, as well as to protruding lamellipodia. siRNA to decrease INF2 protein levels revealed that INF2 KD cells lacked stress fibers in their lamellae and possessed wider lamellipodia. Furthermore, actin filament barbed end localization and photobleach marking of remnant SF in INF2 KD cells showed that INF2 promotes actin polymerization from free barbed ends at FA to form stress fibers. We then determined the role of INF2 in FA functions. Analysis of INF2 and paxillin dynamics showed that INF2 is recruited to FA during elongation, suggesting that it may be required for FA maturation and function. Depletion of INF2 showed that in contrast to growth and elongation of FA in control cells, FA in INF2 KD cells maintained an immature size and shape, yet still contained markers of mature FA including vinculin, zyxin, and tensin. This indicates that INF2 is required for morphological but not compositional maturation of FA. Indeed, although cells lacking INF2 showed only minor defects in migration and traction force generation, they were unable to remodel or generate a dense ECM, indicating that fibrillogenesis requires morphological but not compositional maturation. Collectively, our results show that INF2 is critical to specifying the function of a distinct stress fiber/FA cohort, and reveal the critical role of stress fibers in the maturation of FA to mediate ECM remodeling.

P904**Board Number: B223****Microtubule end-binding protein CLIP170 and formin mDia1 interact to form a barbed end-tracking complex that accelerates actin polymerization.**J.L. Henty-Ridilla¹, A. Rankova², J.A. Eskin², B.L. Goode²;¹Brandeis University, Waltham, MA, ²Department of Biology, Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, MA

Strict coordination of actin and microtubule (MT) dynamics is critical for many cellular processes, including directed cell migration, axon guidance, and cell and tissue morphogenesis. However, the mechanistic basis of actin-MT crosstalk is still not well understood. We identified sequences in CLIP-170 (cytoplasmic linker protein 170) homologous to formin-interacting motifs found in yeast Smy1, which led us to investigate CLIP-170 effects on formin-mediated actin assembly. Purified full-length CLIP-170 increased the rate of mDia1-dependent actin polymerization by 3.5-fold (from ~55 to 180 subunits s⁻¹ μM⁻¹) specifically in the presence of profilin. However, CLIP-170 had no effects on actin dynamics in the absence of mDia1, and failed to regulate the activities of formins Bni1 and Bnr1. Similar activities on mDia1 were observed for all three alternatively spliced isoforms of CLIP-170, suggesting that this formin-regulating function may be universal for CLIP-170. Using multi-wavelength single molecule TIRF microscopy, we observed SNAP-547-mDia1 molecules persisting on the growing ends of actin filaments during CLIP-170-accelerated polymerization. SNAP-649-CLIP-170 molecules transiently associated with mDia1-capped filaments, and correlated with enhanced rates of elongation. CLIP-170 also increased the ability of mDia1 to protect actin filament ends from capping protein. Together, these results suggest that CLIP-170 and mDia1 interact to form barbed-end tracking complexes that support extremely rapid polymerization (0.5 μm s⁻¹ μM⁻¹ profilin-actin) and protect growing actin filament ends from being capped. We are now investigating the effects of microtubule and EB1 binding on CLIP-170-mDia1 activities, and testing whether CLIP-170 coordinates MT and actin polymer dynamics *in vivo*.

P905**Board Number: B224****V-1/Myotrophin Regulates Capping Protein Activity in Vivo.**G. Jung¹, C.J. Alexander*², B. Chen³, E. Betzig³, J.A. Hammer⁴;¹NIH/NHLBI, Bethesda, MD, ²NHLBI, NIH, Bethesda, MD, ³HHMI/Janelia Farm Research Campus, Ashburn, VA, ⁴LCB, NIH/NHLBI, Bethesda, MD

Mammalian myotrophin/V-1 is a ~13 kDa, ankyrin-repeat protein that binds Capping Protein (CP) *in vitro* with an affinity of ~20 nM, creating a 1:1 complex that has no affinity for the barbed end. V-1 has the potential, therefore, to influence actin polymerization *in vivo* by reducing the extent of barbed end capping. *Dictyostelium discoideum* (*D.d.*) contains a single gene encoding a 13 kDa protein with high sequence similarity to mouse V-1. Like mouse V-1, *Dd* V-1 binds CP, is present in cells at a ~four-fold molar excess over CP, and no longer binds CP when a function-blocking point mutation (FBM) is

introduced into the first ankyrin loop (FBM *Dd V-1*). Consistent with V-1's ability to sequester CP, over-expression of *Dd V-1* results in an elevation in total cellular F-actin content that scales positively with the degree of over-expression. Importantly, over-expression of FBM *Dd V-1* does not alter cellular F-actin levels, arguing that the effect of V-1 over-expression is due to its ability to sequester CP. The over-expression of *Dd V-1*, but not FBM-*Dd V-1*, also induces the formation of actin-rich, filopodial-like structures that scales positively with the degree of over-expression. In contrast to over-expression, *Dd V-1* null cells created by homologous recombination exhibit a large decrease in cellular F-actin content. Moreover, these cells exhibit significant decreases in growth rate, macropinocytosis rate, random motility rate, polarity during migration, and chemotactic streaming efficiency. Importantly, these defects are rescued by wild type V-1 but not by FBM *Dd V-1*. Together, these results argue that V-1 plays a major role in regulating actin assembly in cells. Moreover, the fact that FBM V-1 neither induces over expression phenotypes nor rescues null cell phenotypes argues that V-1 exerts its effects on the actin cytoskeleton by buffering cellular CP. We conclude, therefore, that reductions and elevations in cellular CP activity caused by the over-expression and knock out of V-1, respectively, result in changes in cellular actin content that are consistent with CP's barbed end capping activity. Moreover, the functional consequences of these changes, which involve alterations in numerous cellular activities that depend on actin assembly, indicate that V-1 is physiologically important.

P906

Board Number: B225

A Mechanism for Accelerated Actin Filament End-Shortening and Direct Visualization of Rapid Treadmilling.

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A fundamental property of cytoskeletal networks is their ability to be dynamically assembled and disassembled. While microtubules are inherently unstable polymers and abruptly transition to states of rapid depolymerization, actin filaments are inherently stable and undergo relatively slow depolymerization that must be enhanced *in vivo* by other factors. One mechanism for accelerating actin filament turnover is severing by cofilin, but the slow dissociation of subunits from severed filament ends remains rate-limiting. Here, we describe a complementary mechanism, mediated by Twinfilin, which accelerates loss of polymer mass specifically from filament ends. Twinfilin alone causes 3-fold faster shortening at barbed ends of filaments, and working in concert with Srv2/CAP causes 26-fold faster shortening at pointed ends. This end-shortening activity is conserved in evolutionarily distant yeast and mouse homologs of Twinfilin and Srv2/CAP, and specifically acts on 'aged' ADP-F-actin. Single molecule analysis reveals that Twinfilin processively associates with the shortening barbed ends, and also associates with filament sides. Ultrastructural analysis shows that Twinfilin induces major changes in actin filament structure. Finally, accelerated shortening at pointed ends continues even under assembly

conditions, concurrent with barbed end growth, allowing direct visualization of rapid actin filament treadmilling.

P907

Board Number: B226

Modification of VASP architecture reveals a fundamental difference between actin-filament elongation in solution or when clustered on a surface.

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The only proteins known so far that directly enhance filament elongation by interaction with the growing barbed end and recruitment of monomeric actin for polymerization are formins and Ena/VASP proteins. However, despite the relevance of Ena/VASP proteins for many actin-based processes, we in fact still know rather little about their molecular mode of action as compared to formins. Based on previous analyses of chimeras from fast and slow elongating VASP proteins by in vitro TIRF microscopy, kinetic and thermodynamic measurements we showed that the rate of VASP-mediated filament elongation depends primarily on the G-actin recruitment motif (GAB). Based on these experiments we were able to formulate a testable mathematic model of Ena/VASP-mediated actin-filament elongation. It suggests that at steady state one polypeptide chain of the VASP tetramer is bound to the filament terminal subunit by its GAB, leaving a number (N) of free GABs to capture actin monomers from solution for subsequent filament incorporation. Global fitting of the experimental data yielded N=3, suggesting that a single VASP tetramer operates at a growing filament tip. However, previous work revealed that single VASP tetramers are rather poorly processive, as opposed to surface-clustered VASP which allows long lasting and robust filament elongation even in the presence of high concentrations of capping protein. Since the number of free GABs (N) was obtained by fitting, we began experimentally testing the model by manipulation of VASP oligomerization by deleting or replacing the tetramerization motif of the fast elongating hVASP chimera with the oligomerization motifs of different proteins yielding synthetic VASP monomers, dimers, trimers and hexamers. Analyses of these VASP oligomerization mutants in solution showed that the number of free GABs (N) directly correlated with the elongation rate, and is in excellent agreement with model predictions. Interestingly, when clustered in high density on beads, the elongation rate of the same mutants was highly similar suggesting that surface tethered elongation operates at a fixed rate. These findings further indicate that the elongation rate on a surface may be limited to a defined number of contributing GABs, which in turn could be contributed by different VASP molecules and operate together around a central actin filament.

P908**Board Number: B227****Capping Protein Function in Cells requires an interaction with a CPI –motif protein.**M. Edwards¹, P. McConnell², D.A. Schafer³, J.A. Cooper²;¹Cell Biology, Washington University School of Medicine, St. Louis, MO, ²Department of Cell Biology and Physiology, Washington University School of Medicine, Saint Louis, MO, ³University of Virginia, Charlottesville, VA

Capping protein, CP, a ubiquitous heterodimeric actin binding protein, plays an essential role in actin assembly in vivo and in vitro. CP binds the barbed end of actin filaments with high affinity, blocking the addition and loss of actin subunits. Previous models for CP function in cells assumed that CP is constitutively active, diffusing freely to find and cap barbed ends. However, the discovery of CP-binding proteins with CPI (Capping Protein Interacting) motifs raises the possibility that, in order to function properly in cells, CP might need to be targeted to specific cellular locations or have its capping activity decreased.

CPI motifs are found in a diverse group of proteins with the consensus sequence LXHXTXXRPKX₆P. CPI-motif proteins bind CP and decrease but do not abolish its affinity for barbed ends of filaments. They promote the dissociation of bound CP from the barbed end of actin filaments.

Here we tested the activity in cells of a mutant form of CP that cannot bind to the CPI motif. This CP mutant binds and caps barbed ends as well as wild type CP does. However, the mutant displays little to no function in cells; it failed to rescue loss-of-function phenotypes in CP-depleted cells. Overexpression of the CP mutant had a dominant negative effect in cells, phenocopying the depletion of CP from cells. In addition, the CP mutant failed to localize properly in cells.

Our results indicate that, in cells, CP does not function unless it can bind to a CPI-motif protein. The reason that this interaction is essential may be because CP must be targeted to a location and / or that the capping activity of CP must be decreased. Regardless, these findings will require a major change in models for actin assembly in cells.

P909**Board Number: B228****Identification of kinases that phosphorylate Xenopus Slingshot phosphatase during oocyte maturation.**S. Iwase¹, S. Ono², H. Abe¹;¹Nanobiology, Chiba University, Chiba, Japan, ²Pathology, Emory University, Atlanta, GA

We have previously demonstrated that Xenopus Slingshot phosphatase (XSSH), an activator for Xenopus ADF/cofilin, is hyperphosphorylated just after GVBD. This phosphorylation is accompanied by

dephosphorylation of XAC that has been mostly phosphorylated in immature oocytes. This XAC dephosphorylation after GVBD is completely suppressed by latrunculin B, an actin monomer-sequestering drug. On the other hand, jasplakinolide, an F-actin-stabilizing drug, induces dephosphorylation of XAC. Effects of latrunculin B and jasplakinolide are reconstituted in cytostatic factor-arrested extracts (CSF-extracts), and the XAC dephosphorylation is abolished by depletion of XSSH from CSF-extracts, suggesting that XSSH functions as an actin filament sensor to facilitate actin filament dynamics via XAC activation. In this study, in order to clarify the precise role of XSSH phosphorylation, we first explored the kinases that phosphorylate its tail domain during oocyte maturation and found that XSSH is phosphorylated at multiple sites within the tail domain by different kinases during oocyte maturation. The tail domain was divided into two regions, designated as GST-N-tail and GST-C-tail, and each were assayed for mobility shift with CSF-extracts. Although both regions were phosphorylated to represent striking mobility shifts, we found that GST-N-tail was more sensitive to phosphorylation using serially diluted CSF-extracts. This mobility shift of GST-N-tail was mostly suppressed in interphase-extracts but recovered by addition of α N85-cyclin B to the interphase extracts, indicating that the phosphorylation of GST-N-tail occurs specifically at both meiotic and mitotic phases. By a combination of kinase inhibitors, biochemically isolated kinases and site-directed mutagenesis, we conclude that MPF, p90RSK and p38MAPK, which are reported to be deeply involved in oocyte maturation, directly phosphorylate the tail domain, and identified their phosphorylation sites in the N-tail region.

P910

Board Number: B229

Dynamic regulation of actin filaments by ADF/cofilin is required for the assembly of microtubule-organizing center and meiotic spindles in *Xenopus laevis* oocytes.

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Oocyte maturation is defined by resumption of meiosis to release oocytes from arrest in meiotic prophase I. This process starts with the breakdown of the nuclear envelope of the germinal vesicle, a giant nucleus specifically formed in oocytes, (i.e., germinal vesicle breakdown (GVBD) or nuclear envelope breakdown). In *Xenopus* oocytes, a disc-shaped organelle called the microtubule-organizing center and transient microtubule array (MTOC-TMA) assembles at GVBD to capture chromosomes and transport them to the animal cortex. Thereafter, the MTOC-TMA disappears and chromosomes are aligned on the spindle microtubules. We previously demonstrated that phosphorylation of *Xenopus* Slingshot (XSSH), a *Xenopus* ADF/cofilin (XAC)-specific phosphatase, at multiple sites within the tail domain occurs immediately after GVBD and is accompanied by dephosphorylation of XAC that has been mostly phosphorylated in immature oocytes. Injection of the anti-XSSH antibody, which blocks full phosphorylation of XSSH after GVBD, inhibits both meiotic spindle formation and XAC dephosphorylation. Co-injection of the constitutively active XAC with the antibody suppresses this phenotype. Treatment of oocytes with jasplakinolide also impairs spindle formation. These results strongly suggest that elevation of actin dynamics by XAC activation through XSSH phosphorylation is

required for the meiotic spindle assembly in *Xenopus laevis*. However, the precise role of XAC on cellular actin organization that regulates microtubule structures has still remained unclear. In this study, we show that the orderly formation of microtubule structures requires dynamic reorganization of cytoplasmic and intranuclear actin filaments by XAC regulated through XSSH. Surrounding the germinal vesicle (GV) in immature oocytes, cytoplasmic actin filaments reorganized to accumulate beneath the vegetal side of GV, where MTOC-TMA assembled, just before GV breakdown (GVBD). Immediately after GVBD, MTOC-TMA developed well and intranuclear actin filaments began to disassemble from the vegetal to animal side. Suppression of XAC dephosphorylation by anti-XSSH antibody injection inhibited these actin filament dynamics. Interestingly, antibody-injected oocytes lacked proper formation and localization of MTOC-TMA and meiotic spindles. We further confirmed that while both XAC and XSSH are diffusely distributed throughout the cytoplasm, only XAC is exclusively absent from the base region of MTOC-TMA where cytoplasmic actin filaments have already assembled. As constitutively active S3A-cofilin-injection could disrupt cytoplasmic actin filaments and MTOC-TMA, the cytoplasmic actin filaments associated once with MTOC could be protected from XAC under regulation. Taken together, XAC appears to reorganize cytoplasmic actin filaments required for precise assembly of MTOC and to regulate intranuclear actin filament dynamics essential for the formation of meiotic spindles.

P911

Board Number: B230

Cellular chirality arising from the self-organization of the actin cytoskeleton.

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The development of a chiral pattern of actomyosin was revealed by studying the self-organization of the actin cytoskeleton in human fibroblasts confined to cell-sized circular adhesive islands. We identified several distinct stages of actin cytoskeleton evolution, starting with the formation of a radially symmetrical system of actin bundles. Myosin-II-deficient alpha-actinin-enriched radial fibers grew centripetally from peripheral focal adhesions, while myosin-IIA-enriched transverse fibers moved centripetally along radial fibers. Formin inhibitor blocked the formation of radial fibers while myosin II inhibitor suppressed formation of both radial and transverse fibers. This radial pattern evolved spontaneously into the chiral pattern as a result of the synchronous and unidirectional tilting of all radial fibers. Subsequently transverse fiber movement acquires a tangential component. We propose a novel mechanism of myosin IIA-driven retrograde flow where contractile stresses within transverse fibers drive their movement along radial fibers. Computational modeling demonstrated that this mechanism can explain the pattern transition from radial to chiral. Remarkably, the chiral pattern is characterized by

a defined handedness. This directionality could be reversed by overexpressing full-length alpha-actinin-1. Expression of a truncated alpha-actinin-1 construct lacking actin binding domain abolished formation of radial fibers, but resulted in the formation of a new type of long actin fibers containing myosin IIA and anchored at focal adhesions. These actin fibers also tilt unidirectionally, preserving the handedness of the direction of tilt similarly to the radial fibers in control cells. Expression of an alpha-actinin fragment containing the integrin-binding site also perturbed the development of radial fibers. Depletion of alpha-actinin-1 via siRNAs resulted in phenotypes analogous to those observed in cells expressing these dominant-negative alpha-actinin-1 constructs. Taken together, these results suggest that while the crosslinking function of alpha-actinin-1 is not essential for the initiation of the chiral asymmetry of the actin cytoskeleton, it is essential for the alteration of the chirality seen in alpha-actinin-1 overexpressing cells. We hypothesize that the handedness of the chiral pattern depends on the intrinsic helical symmetry of actin filaments, which is transformed (with the aid of formin and alpha-actinin-1) into a unidirectional torque that triggers the transition of unstable radial system to chiral rotation. Thus, self-organization of the actin cytoskeleton can provide built-in machinery that potentially allows cells to distinguish between left and right.

P912

Board Number: B231

Traffic Jam regulates actin protrusions of migrating border cells through PKC δ in the *Drosophila* ovary.

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The migrating border cell cluster (BCC) in the *Drosophila* ovary is a simple, genetically-tractable system to study cell migration in vivo. The BCC, which contains 6-8 migratory cells, invades the germline cyst and migrates toward the oocyte during mid-oogenesis. To generate the force needed to push the cluster forward, extension of actin-based cellular protrusions by the leading cell is crucial. Not surprisingly, a number of actin-regulating factors have been shown to be necessary for proper BCC motility. Our previous work identified the *Drosophila* large Maf transcription factor Traffic Jam (Tj) as an important regulator of BCC migration. We recently discovered that Tj acts as a negative regulator of actin protrusions in the BCC. Increased Tj expression leads to a significant decrease in the number of prominent protrusions extended by the BCC, which in turn causes a severe delay of BCC migration. In contrast, reduced Tj expression causes cells within the BCC to extend extra protrusions in multiple directions. To find downstream targets of Tj that regulate the actin cytoskeleton, we conducted a microarray screen using RNA from isolated border cells with normal, reduced, and increased Tj activity. *Drosophila* Protein Kinase C δ (PKC δ), a serine/threonine kinase, was identified as a putative activation target of Tj in this screen. Similar to Tj, altering PKC δ expression leads to abnormal actin organization: PKC δ loss-of-function causes an increased number of radially-arranged protrusions, whereas PKC δ gain-of-function leads to a decreased number of prominent actin protrusions. Analysis of the epistatic

relationship between Tj and PKC δ indicates that PKC δ is an important downstream factor through which Tj exerts its effects on actin. We further identified a putative target of PKC δ activity, the actin-polymerizing factor Enabled (Ena)/VASP, which is known to be important for BCC migration. Ena is normally localized cortically along the periphery of the cluster, and enriched in the leading protrusion. Tj or PKC δ reduction leads to mis-localization of Ena to the cytoplasm and failure of Ena to be enriched in the protrusions. Overexpression of Tj or PKC δ seems to cause stronger and more punctate cortical localization of Ena. Based on our data, we propose a new pathway needed to regulate actin dynamics in the BCC: the transcription factor Tj directly or indirectly upregulates the expression of the kinase PKC δ , which is needed for proper formation of actin protrusions, a function that might be mediated through proper localization of the actin-regulating factor Ena.

P913

Board Number: B232

Prostaglandins temporally regulate actin remodeling during *Drosophila* oogenesis.

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Actin remodeling must be tightly regulated during both development and adult tissue homeostasis. While prostaglandins (PGs), lipid signals produced downstream of cyclooxygenase (COX) enzymes, are known to regulate actin dynamics in cultured cells and platelets, their roles during development remain largely unknown. Previously, we identified the *Drosophila* COX-like enzyme, Pxt, and found that loss of Pxt causes numerous actin remodeling defects during late-stage oogenesis. Here, we define a new role for PG signaling during oogenesis—temporal restriction of actin remodeling. Loss of Pxt results in the early onset of actin remodeling, causing the formation of extensive actin filaments and aggregates in the posterior nurse cells of stage 9 (S9) follicles. Wild-type follicles exhibit similar structures at a low frequency. We performed a screen to identify actin-binding proteins that localize to early actin structures in *pxt* mutant, but not wild-type, S9 follicles in order to identify the factor(s) responsible for promoting early actin remodeling in the absence of PG signaling. Using this approach, we found that Enabled (Ena), an actin elongation factor, preferentially localizes to early actin structures in *pxt* mutants, suggesting that PG signaling is required to restrict Ena localization/activity during S9. Consistent with this model, we find that reduced levels of *ena* are sufficient to strongly suppress early actin remodeling in *pxt* mutants. Additionally, a parallel pharmaco-genetic interaction screen revealed that heterozygous loss of either *ena* or its functional antagonist *capping protein*, strongly sensitizes follicles to the effects of COX inhibition during S10B. Consistent with these results, we find that Ena localization to the barbed ends of actin bundles during S10B is reduced in *pxt* mutants. Together, these data lead us to propose a model in which PG signaling temporally regulates actin remodeling during *Drosophila* oogenesis, in part,

by differentially controlling Ena localization/activity, such that PG signaling inhibits Ena-dependent actin remodeling during S9, while promoting it during S10B. As PG-dependent regulation of Ena's mammalian homologs (Mena, EVL, and VASP) is poorly understood, *Drosophila* oogenesis provides an ideal system to elucidate the conserved mechanisms by which PG signaling regulates this family of proteins to temporally control actin remodeling.

P914

Board Number: B233

Abiotic fungal biofilms of *C. albicans* secrete factors that remodel the actin cytoskeleton in keratinocytes and fibroblasts.

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Candida albicans is an opportunistic fungal pathogen that causes candidiasis in humans. Many infections caused by *C. albicans* are a direct consequence of its ability to form biofilms. The intracellular signaling pathways that are triggered in human epithelial cells by the adhesion of *C. albicans* and by secreted virulence factors released by biofilms are poorly understood. In a previous study, we showed that the virulence factors, secreted aspartyl proteases (Saps), produced by *C. albicans* target the actin cytoskeleton and its associated proteins in keratinocytes. In this study, a new *in vitro* assay was developed to determine the virulence of medically relevant *C. albicans* biofilms and evaluate changes of the actin cytoskeleton in human keratinocytes and mouse fibroblasts in the presence of these biofilms. Abiotic fungal biofilms were created by addition of yeast cells obtained from overnight *C. albicans* cultures to cell culture dishes followed by incubation in keratinocyte serum free medium for 3 hours. We used OKF6/TERT-2 cells, an immortalized cell line of human oral keratinocytes and 3T3 fibroblasts co-cultured with hyphae-forming wild-type *C. albicans* (SC5314) and *efg1D/efg1D* and *sap4-6D/sap4-6D* mutants. The actin remodeling was determined at 0.5, 1, 2 and 3 hours by high-resolution fluorescence microscopy and quantitative image analysis. OKF6/TERT-2 cells and 3T3 fibroblasts exposed to biofilms formed by wild-type *C. albicans* showed a dynamic remodeling of the actin cytoskeleton compared to untreated cultures. Coculture of OKF6/TERT-2 cells with *C. albicans* stimulated the formation of lamellipodia at 1 hour followed by the accumulation of actin retraction fibers at the cell edges between 2 and 3 hours. In addition, an increase in the formation of cell-cell adhesions was observed after 1 hour and newly formed cell-cell adhesions began to disintegrate after 3 hours. Coculture of 3T3 fibroblasts with *C. albicans* resulted in the formation of actin retraction fibers and in the decrease of focal adhesions. 80-90% of all cells exhibited this behavior. Interestingly, we did not detect internalized hyphae with OKF6/TERT-2 cells during coculture with *C. albicans*. The latter observation suggests that changes in the actin cytoskeleton and its associated proteins are primarily caused by secreted virulence factors by *C. albicans*. *C. albicans* mutants *sap4-6D/sap4-6D* and *efg1D/efg1D* with diminished virulence and reduced abilities to form biofilms were found to elicit attenuated changes in keratinocyte and fibroblasts responses compared to those that were cocultured with wild-type *C. albicans*. The induction of actin remodeling in keratinocytes by pathogenic *C. albicans* may constitute an important first step in

the invasion mechanism and these observations could lead to improvements in the prevention and treatment of biofilm-based infections.

P915

Board Number: B234

Reciprocal regulation of actin remodelling and cell migration by Calcium and Zinc: role of H₂O₂ and TRPM2 channels.

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H₂O₂ is a signalling molecule thought to play a role in cell migration by remodelling actin cytoskeleton. However, the mechanisms remain unclear. Here, we investigated the role of the H₂O₂-sensitive transient receptor potential-melastatin 2 (TRPM2) cation channel in cell migration using the HeLa and PC-3 cell lines. Dynamics of actin cytoskeleton and focal adhesions that regulate cell migration were monitored using confocal microscopy. Actin was stained with Alexa⁴⁸⁸-phalloidin, and focal adhesions with anti-paxillin antibodies. Cell migration was monitored using the agarose spot assay, where the agarose spot contained test substances. Exposure of cells to H₂O₂ caused filopodia-like protrusions, loss of stress fibres and disassembly of focal adhesions. Consistent with these changes, H₂O₂ promoted cell migration. Inhibition of TRPM2 channels with pharmacological agents and small interference RNA targeted to TRPM2 channels prevented all cellular changes induced by H₂O₂. H₂O₂ activation of TRPM2 channels increased the cytosolic levels of both Ca²⁺ and Zn²⁺. To distinguish the role of individual ions, we raised the intracellular levels of Ca²⁺ and Zn²⁺ using ionophores, A23187 and pyrithione respectively. The results showed remarkable differences in how Ca²⁺ and Zn²⁺ affect the cytoskeleton and cell migration: Zn²⁺ promoted filopodia-like protrusions, loss of stress fibres, disassembly of focal adhesions and cell migration, while Ca²⁺ displayed the complete opposite effects. Thus, our findings demonstrate a novel role for TRPM2 channels in cell migration where Zn²⁺ and Ca²⁺ affect the actin cytoskeleton and focal adhesions in a reciprocal fashion.

P917**Board Number: B236****Wave-like actin dynamics for contact guidance.**W. Losert¹, C. Guven², S. Das², X. Sun³, A. Carlsson⁴, J. Fourkas³;¹Dept of Physics, University of Maryland, College Park, MD, ²Dept of Physics and Institute for Physical Science and Technology, University of Maryland, College Park, MD, ³Dept of Chemistry and Biochemistry, University of Maryland, College Park, MD, ⁴Physics, Washington University School of Medicine, St Louis, MO

How do cells sense and follow the texture of their surrounding environment in the natural world? In rapidly moving cells, *Dictyostelium* and neutrophils, we discovered that cells utilize actin waves to sense and follow surface nanotopography. The wave-like character of the sensing allows cells to follow topographical features on the 100s of nm scale, larger than proteins or macromolecular clusters that are involved in other sensing tasks.

Specifically, cells use actin polymerization waves that propagate isotropically in eukaryotic cells when migrating on flat surfaces. We find that exposing cells to nanofabricated ridges hundreds of nm wide breaks the symmetry of actin waves and results in guided polymerization. We present quantitative measurements of the speed and orientation of actin waves generated in *Dictyostelium discoideum* cells and neutrophils in contact with such nanoscale ridges for a broad range of conditions. We find that spatially periodic, ~300-nm wide nanoridges spaced ~1.5 microns apart, slightly larger than typical actin wave sizes, lead to the best contact guidance of the migrating cells. Through measurements of the distribution of actin-wave speeds and directions relative to the nanoridges we show that actin polymerization is preferentially localized to nanoridges over a range of spatial periodicities, and that the speed of actin polymerization waves is increased along the nanoridges.

A stochastic growth model of actin polymerization dynamics reproduces these key observations, if nanoridges are modeled as locally increasing the concentration of nucleation promoting factors. We use this model together with experiments to demonstrate that the propagation speed of actin waves due to the underlying nanotopography decreases when the concentration of actin monomers decreases. We show that the effect of nanoridges on actin polymerization can be modeled as a local positive feedback on the actin polymerization machinery.

Our proposed actin based dynamic contact guidance mechanism utilizes ubiquitous cytoskeletal machinery and may thus be operational in a broad range of cell types and natural contexts. Part of this work appeared in *ACS Nano* **8**, 3546, (2014).

P918**Board Number: B237****A quantitative, FRET-based method for observing changes in non-muscle myosin II phosphorylation in living cells.**M.L. Markwardt¹, M.A. Rizzo¹;¹Physiology, University of Maryland School of Medicine, Baltimore, MD

Phosphorylation of the non-muscle myosin II regulatory light chain (RLC) enables association of myosin fibers with the actin cytoskeleton and filament contraction. Tight spatial and temporal regulation of RLC phosphorylation is thus necessary to generate vectorial force during cell migration. Here we report a method for observing, and quantifying, phosphorylation-induced changes in myosin II conformation in living cells using fluorescent protein-tagged RLC. In the absence of phosphorylation, we observe fluorescence resonance energy transfer (FRET) between tagged-RLCs using fluorescence polarization microscopy. Homotransfer decreases upon stimulation of RLC phosphorylation by plating cells on fibronectin. Similar changes in homotransfer are observed by mutating RLC phosphorylation sites to aspartate, and inhibited by mutation of these same sites to alanines. Further, low-FRET (phosphorylated) cellular regions co-localize with phospho-RLC antibody staining. These results are consistent with FRET-based reporting of phosphorylation-induced RLC conformational changes. Dynamic changes were observed in response to myosin-light chain kinase activation, via application of KCl in cells co-expressing mCherry-RLC and an optimized MLCK reporter containing mCerulean3. Addition of nitric oxide to stimulate myosin phosphatase increased myosin-RLC FRET, consistent with promotion of RLC dephosphorylation. Further, we observed changes in RLC FRET consistent with phosphorylation on the leading edge of spreading membranes, and FRET changes consistent with RLC dephosphorylation prior to retraction of cellular structures. These studies demonstrate the utility of a single color FRET-based approach for resolving spatio-temporal changes in non-muscle myosin II phosphorylation states in living cells.

P919**Board Number: B238****TRPM4 channels are required for biomechanical remodeling during wound healing.**L. Ortiz¹, T. Recabarren^{1,2}, A. Colombo², E. Leiva-Salcedo¹, A. Romero¹, J. Rivas¹, I. Silva¹, G. Flores¹, A. Álvarez¹, M. Cáceres¹, O. Cerda¹;¹Programa de Biología Celular y Molecular, Facultad de Medicina, Universidad de Chile, Santiago, Chile,²Programa de Anatomía y Biología del Desarrollo, Facultad de Medicina, Universidad de Chile, Santiago, Chile

Cellular migration and contractility are fundamental processes in wound healing that are regulated by a variety of concerted mechanisms such as cytoskeleton rearrangements, focal adhesion turnover, protein

kinase/phosphatase activities, and Ca^{2+} oscillations. TRPM4 is a Ca^{2+} -activated non-selective cationic channel (Ca^{2+} -NSCC) that conducts monovalent cations and localizes at focal adhesion complexes. Here, we show that suppression of TRPM4 in MEFs impacts turnover of focal adhesions, serum-induced Ca^{2+} influx, focal adhesion kinase (FAK) activity, and results in reduced cellular spreading, migration and contractile behavior *via* regulation of FAK and Rac GTPase activities. Finally, we demonstrate that TRPM4 activity regulates cellular contractility and migration *in vivo*, impacting morphogenetic movements during development and wound closure in zebrafish, and regulating the re-epithelialization and granulation tissue formation during cutaneous wound healing in mice. Together, these findings provide the first evidence for a TRP channel specifically localized to focal adhesions, where it performs a central role in modulating cellular migration, contractility and biomechanical remodeling during development and wound healing.

P920

Board Number: B239

Actin depolymerisation and crosslinking join forces with myosin II to drive secretion via compression of fused secretory vesicles.

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In recent years it has become evident that actin-based forces are required for secretion of poorly soluble vesicle contents. It is now well established that actin and myosin are specifically recruited to the surface of exocytic vesicles following fusion with the plasma membrane and promote active extrusion of cargo. Yet, little is known about the molecular mechanisms that regulate actin coat formation and drive coat contraction on fused granules. We have recently demonstrated that actomyosin dependent compression of fused lamellar bodies (LBs) is essential to drive secretion of pulmonary surfactant. Here we provide a detailed kinetic analysis of the molecules regulating actin coat contraction on fused LBs. We demonstrate that Rock1 and myosin light chain kinase (MLCK) translocate to fused LBs and activate myosin II on actin coats. Yet, myosin II activity is not sufficient for efficient actin coat contraction. In addition, Rock1 regulates cofilin-1 activity. Regulated actin depolymerisation by cofilin-1 and actin crosslinking via α -actinin is essential for full contraction of the actin coat. In summary, our data support a model in which actin depolymerisation and crosslinking join forces with myosin II to contract actin coats around fused secretory vesicles to drive secretion.

Myosin Motors 2

P921

Board Number: B241

14-3-3 proteins tune non-muscle myosin II assembly, providing a possible bridge between cell mechanics and cancer metastasis.

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The 14-3-3 family comprises a group of small acidic regulatory proteins which are essential, ubiquitous, and highly conserved across eukaryotic species. These proteins have roles in numerous core cellular processes such as DNA damage repair, cell cycle control, apoptosis prevention, and certain signaling pathways. Generally 14-3-3s act as tumor suppressors and consequently, are down-regulated in many cancers. By contrast, overexpression of 14-3-3s sigma, epsilon, zeta, and eta correlate with a higher metastatic potential and poorer clinical outcomes in breast, liver, and pancreatic cancers. The literature has no unified theory on how 14-3-3 proteins that are normally tumor suppressors become oncogenes. However, we have uncovered a role for 14-3-3s in regulating the assembly of non-muscle myosin II. This demonstrates that 14-3-3s could tune cell mechanics directly, and therefore, contribute to the progression of metastatic cancers. Here, we examine the entire landscape of myosin II assembly regulation by 14-3-3 in *Dictyostelium* (one 14-3-3, one non-muscle myosin II) and humans (seven 14-3-3s and three non-muscle myosin IIs). In *Dictyostelium*, 14-3-3 mediates a pathway between microtubules and racE small GTPase to regulate myosin II assembly, and 14-3-3's expression levels negatively correlate with BTF accumulation. *In vitro* assembly assays using purified myosin II tail fragments and 14-3-3 demonstrate that this interaction is direct and phosphorylation-independent. We then extended our analysis to the seven human 14-3-3 paralogs and the three non-muscle myosin IIs. We found that the seven human paralogs of 14-3-3 affect the assembly of myosin II filaments in different ways, broadly clustering into those that cause myosin II overassembly and those that cause its solubilization. Assemblers and solubilizers can directly compete to govern the overall level of myosin II assembly. Examining assembled myosin II filaments by EM confirms that the average filament size correlates with the overall assembly level. Furthermore, we mapped three critical residues which differ between the two classes and discovered that mutating any of these residues converts an assembler to a solubilizer. Our findings demonstrate a novel phosphorylation-independent method for regulating myosin II assembly that is mechanistically conserved from amoebas to humans. These findings imply that altered 14-3-3 expression profiles could directly modulate cell mechanics in metastatic cancers, which would be of great interest for basic and clinical sciences alike.

P922**Board Number: B242****Counting molecules in non-muscle myosin II filaments.**X. Wan¹, C. Patino Descovich¹, D.A. Applewhite¹, S. Rogers¹, P.S. Maddox¹, A.S. Maddox¹;¹Department of Biology, University of North Carolina, Chapel Hill, NC

Non-muscle (NM) myosin II is involved in many important cellular processes, including migration, adhesion and cytokinesis. Like its muscle counterpart, NM myosin II assembles into bipolar filaments; this multimerization is thought to be essential for acto-myosin contractility. The classical sliding filament hypothesis for cytokinesis states that NM myosin II acts in cytokinesis by sliding actin. However, recent studies implicate its actin crosslinking role as being more important for cytokinesis. Since each head in a myosin filament can bind F-actin, the number of heads per filament will affect the balance among its bundling, crosslinking and sliding activities, as well as the load-dependent feedback on these activities. Electron microscopy studies of interphase cells from different species have shown NM myosin II filaments contain 16-30 heads, but the size of filaments has never been determined in cytokinesis.

We first studied the number of NM-myosin II in meiotic and mitotic *C. elegans* zygotes. GFP was tagged at the tail of NM myosin heavy chain, so that the fluorescence from the tags on all myosin molecules in a filament contributed to a single diffraction-limited focus. Myosin was imaged by TIRF microscopy until the fluorescence signal was completely bleached. We developed a novel, yet very simple approach to measure the number of bleaching events in the fluorescence decay curves from cells with different level of GFP expression. We found that the total number of myosin molecules in a single filament is 100 – 150. As an independent assessment of myosin filament size, we also measured filament length in cultured *Drosophila* cells using super-resolution microscopy. We measured the distance between diffraction-limited foci containing GFP-tagged regulatory light chain (RLC) at the two ends of myosin filaments, with nm accuracy. On average, myosin filaments are approximately 380 nm long. This method also allowed us to determine the orientation and size of the RLC myosin head foci. Interestingly, increased myosin filament length correlated with more parallel filament head foci. We will combine these measurement techniques with genetic perturbations to understand how NM myosin II filament assembly is controlled and how interaction with actin influences myosin filament structure.

P923**Board Number: B243****Fission yeast UCS protein Rng3p provides surveillance of myosin-II motor function during cytokinesis.**L.W. Pollard¹, B.C. Stark¹, V. Sirotkin², M.J. Lord¹;¹Molecular Physiology and Biophysics, University of Vermont, Burlington, VT, ²Department of Cell and Developmental Biology, SUNY Upstate Medical University, Syracuse, NY

UCS (Unc-45-/Cro1p-/She4p-related) proteins are known to participate in the folding and maturation of myosin-II motors. Fission yeast myosin-II (Myo2p) and UCS protein Rng3p are essential for actomyosin ring formation and cytokinesis. Previous studies suggested that Rng3p is a chaperone for Myo2p given that Rng3p function is critical for the isolation of active Myo2p from fission yeast. Here we employed a combination of in vivo and in vitro studies to understand how Rng3p regulates Myo2p and actomyosin ring function during cytokinesis. Rng3p localizes transiently to both pre-assembled and assembled rings where it contributes to ring assembly, constriction, and Myo2p turnover at the ring. Elevation of Myo2p expression levels influences Rng3p localization and suppresses cytokinesis defects associated with loss of Rng3p function, indicating that Myo2p is the key substrate for Rng3p. We engineered a Myo2p-Rng3p fusion to enforce constitutive association of Rng3p with its substrate in vivo. While the fusion supports growth, it led to obvious morphological defects in cytokinesis characterized by significantly reduced rates of ring assembly, constriction, and disassembly. Current in vitro mechanistic studies are centered on understanding the potential role of Rng3p in destabilization of aged Myo2p motors. Collectively our studies point to a critical role for Rng3p in Myo2p motor homeostasis in the cell.

P924**Board Number: B244****Motor activity is required for stacking of non-muscle myosin II filaments.**A.M. Fenix¹, J. Lewis¹, D.T. Burnette¹;¹Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN

The molecular motor, myosin II, is best known for driving muscle contraction. Myosin II is a hexamer comprised of 2 heavy chains, 2 regulatory light chains, and 2 essential light chains. The two heavy chains create a C-terminal coiled-coil domain forming a rod on one end of the molecule and two N-terminal motor domains on the other end. Myosin II molecules form bipolar contractile-competent filaments by binding each other through their rod domains. In skeletal muscle, myosin II filaments are organized into regularly spaced stacks within sarcomeres. This organization facilitates efficient muscle contraction. However, how stacks of myosin II filaments develop is not well understood. We have recently used structured illumination microscopy (SIM) to resolve individual motor domains of non-muscle myosin IIA filaments in live U2OS cells to reveal that filament stacks result from the expansion of a single filament. Here we use this system as a model to test if the motor activity of myosin II is required for filament stack formation. Previous studies have reported that treatment with relatively high doses (e.g., 50 μ m) of the

myosin II ATPase inhibitor, blebbistatin, dramatically reduces the number of myosin II filaments in both U2OS and other cell types. We sought to find a concentration of blebbistatin that reduced stack height but kept the number of filaments relatively the same. After allowing U2OS cells to spread for 2 hours on Laminin, we treated cells for 1 hour with different concentrations of blebbistatin and then labeled endogenous myosin IIA filaments by immuno-fluorescence. We found that cells treated with 1 μ m and 10 μ m concentrations of blebbistatin had similar number of myosin II filaments as controls cells. However, there was a marked reduction in stack height after treatment. Specifically, there was a 49.3 +/- 13.7% and 34.8 +/-14.1% reduction in the number of stacked myosin II filaments in 10 μ m and 1 μ m blebbistatin treated cells, respectively. Our results suggest that the expansion of a myosin II filament into a stack is more sensitive to changes in motor activity than the initial filament formation.

P925

Board Number: B245

EGF-Mediated Regulation of Myosin-IIA Heavy Chain Phosphorylation Mediates Tumor Extravasation.

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EGF stimulation of MDA-MB-231 breast cancer cells results in the transient phosphorylation of S1943 on the nonmuscle myosin-IIA heavy chain (NMHC-IIA), which enhances myosin-IIA filament disassembly and promotes cell migration in vitro. Using reverse phase protein arrays (RPPAs) and a phosphospecific antibody against S1943, we have extended these observations to show that EGF-stimulated NMHC-IIA S1943 phosphorylation occurs in multiple breast cancer cell lines. In addition, immunohistochemical studies indicate that S1943 phosphorylation is detected at the invasive edge of human breast tumors. To test the role of NMHC-IIA in tumor metastasis, we produced MDA-MB-231 breast cancer cells expressing wild-type, phosphomimetic (S1943E) and non-phosphorylatable (S1943A) mutants. Expression of the S1943E NMHC-IIA markedly increased metastasis in an experimental metastasis assay. Cell biological studies demonstrated that NMHC-IIA S1943 phosphomimetics (S1943 D/E) enhance myosin-IIA filament turnover in vivo and inhibit EGF receptor internalization. In addition, EGF-stimulated S1943 phosphorylation is blocked by inhibitors of Class IA PI 3-kinases as well as inhibitors of casein kinase 2. Altogether, these data suggest that EGF-mediated phosphorylation of NMHC-IIA is linked to invasive behavior and tumor metastasis, and suggest that NMHC-IIA S9143 phosphorylation might serve as a diagnostic marker for metastatic tumors.

P926**Board Number: B246****Conditional Deletion of Nonmuscle Myosin II-A Leads to Squamous Cell Carcinoma of the Tongue.**M. Conti¹, A. Saleh², S. Cornelius², C. Van Waes², R.S. Adelstein¹;¹Laboratory of Molecular Cardiology, Genetics and Developmental Biology Center, NHLBI/NIH, Bethesda, MD, ²Clinical Genomics Unit and Tumor Biology Section, Head and Neck Surgery Branch, NIDCD/NIH, Bethesda, MD

In an effort to investigate the contribution of nonmuscle myosin II-A (NMII-A) to early cardiac development we crossed Myh9 floxed mice and Nkx2.5 cre recombinase mice. Nkx2.5 is expressed in the early heart and later in the tongue epithelium and in a mosaic pattern in the stomach, and spleen. Mice homozygous for deletion of NM II-A (A^{Nkx}/A^{Nkx}) were born at the expected ratio but were 50% smaller than control littermates probably due to insufficient nutrition. Although the hearts developed normally, the conditionally deleted mice developed invasive squamous cell carcinoma (SCC) of the tongue as early as embryonic day 17.5. In general the mice survived for 2-3 months at which time they were sacrificed for humane reasons due to a failure to thrive. In some cases the SCC progressed slowly and the mice survived for up to 2 years of age. In order to assess the reproducibility of these results a second, independent line of Aflox mice was derived from the original, targeted embryonic stem cells. The homozygous floxed mice expressing cre in this second line were also smaller than control littermates and also developed a SCC that was indistinguishable from that of the original cell line and showed a similar timing of tumor formation and lethality. The same phenotype was observed in mice in which one allele was null for II-A and the other allele conditionally ablated by Nkx 2.5 cre expression. In an effort to rescue the phenotype by expression of a second NMHC, mice were generated in which one allele of NMHC II-A was replaced by NMHC II-B under control of the NMHC II-A promoter, but these mice also developed tumors of the tongue. Schramek et al. (Science. 2014, 343, 309-13) have reported that RNAi mediated deletion of NMII-A leads to SCC of the skin in a tumor susceptible background by a mechanism involving p53 stabilization. In our A^{Nkx}/A^{Nkx} mouse tongue epithelium, genetic deletion of NMII-A does not affect the stabilization of p53 in the nucleus after gamma irradiation of mice. Additionally, an in vitro FRET based assay of p53 activation and binding to p21 target sequences in human UMSCC-1 and UMSCC-74A cell lines indicates that siRNA-mediated deletion of II-A itself induces p53 activation. This activation is further enhanced in the presence of DNA damaging agents that induce a p53 response.

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P927**Board Number: B247****Non-muscle myosin IIB is critical for nuclear translocation during 3D invasion.**D.G. Thomas¹, T.T. Egelhoff¹, J. Lammerding², C.M. Denais³;¹Cellular and Molecular Medicine, Cleveland Clinic Lerner Research Institute, Cleveland, OH,²Department of Biomedical Engineering, Cornell University, Ithaca, NY, ³Weill Institute, Cornell University, Ithaca, NY

Tumor metastasis is a complex process that involves the migration of a tumor cell from the primary tumor to a secondary site, involving the formation of leading edge protrusions and the translocation of a bulky nucleus through restrictive spaces. A major component to cellular migration is the motor protein, non-muscle myosin II (NMII). NMII is expressed in all cell types to regulate cellular structure and cytoskeletal dynamics during migration. There are three isoforms of the heavy chain (IIA, IIB and IIC), encoded by three separate genes. Treatment of the murine epithelial cell line, NMuMG, with TGF β 1 induces an increased invasive behavior as well as a dramatic up-regulation of NMIIB at both mRNA transcript and protein levels. This observation led us to investigate NMIIB's role in invasion of tumor cells through a three-dimensional collagen matrix. We used a collagen invasion assay to enhance migration through a collagen gel. In this assay, knockdown of either NMIIA or NMIIB via shRNA caused a dramatic reduction in invasion compared to control, in both the human breast carcinoma cell line MDA-MB 231 and the murine mammary tumor line 4T1. We then analyzed NMIIB's role in nuclear translocation through restrictive spaces. MDA-MB 231 cells were analyzed in three-dimensional chemotaxis chambers that contain 5 micron constriction points. Knockdown of NMIIB but not NMIIA resulted in an increase in the nuclear transit time through constriction points. These data suggest a role for NMIIB in squeezing the nucleus through resistive openings. Rescue of NMIIB expression in the NMIIB shRNA background with a GFP-NMIIB fusion construct restored collagen invasion and normal nuclear translocation rates. Furthermore, perinuclear stress fibers of GFP-NMIIB were visible as the nucleus passed through the 5 micron constrictions. We then tested if NMIIB is essential for the development of traction forces required to constrict the nucleus. During spreading on polyacrylamide gel coated with 50 μ g/ml fibronectin, knockdown of NMIIA but not NMIIB ablates traction force generation during the first hour of spreading. To control for active migration and protrusions, fibronectin was patterned in 30 μ m² grids on polyacrylamide and traction forces were measured after 1 and 16 hours. NMIIB shRNA had modest effect on traction force at one hour compared to control, but dramatically reduced traction force at 16 hours. The converse was true for NMIIA shRNA, where traction force was greatly diminished at 1 hour, but normal at 16 hours. These data suggest that NMIIA is involved in generating traction force during early stage active spreading and protrusion formation, while NMIIB is involved in generation of long-term traction stress in a static condition to maintain cell morphology.

P928**Board Number: B248****Tuning myosin driven sorting on cellular actin networks.**R.F. Hariadi¹, R. Sommese¹, S. Sivaramakrishnan¹;¹Cell and Developmental Biology, University of Michigan, Ann Arbor, MI

Unconventional myosin function in cellular processes emerges from the interaction of multiple myosins on an organelle scaffold with the complex actin cytoskeleton. Currently, the role of myosins in membrane sorting has been extensively studied in reconstituted systems, but these have focused almost exclusively on understanding single actin-myosin interactions. To bridge the gap between single molecule and cellular function, we pair DNA origami scaffolds, containing a defined number of antagonistic myosins, with a model cellular actin network. Scaffolds with antagonistic myosins (V and VI) exhibit unidirectional motion, unlike the bi-directional movement previously reported for groups of kinesin and dynein. For scaffolds with equal numbers of myosin V and VI, minus and plus-end directed movement is equally favored. This two-dimensional flux is significantly lower than that of single filament, emphasizing the importance of actin architecture on myosin behavior. In ensembles with asymmetric motor composition, the net flux of trajectories can be finely tuned by the relative number of myosin V and myosin VI. Thus, modulating the relative number of motor engagement sites represents an elegant sorting mechanism in cells. Finally, the net flux is dependent on the structural properties of the myosin lever arm. Our study demonstrates a novel regulatory function for the myosin lever arm beyond its canonical function as a structural amplifier in the chemomechanical cycle.

P929**Board Number: B249****Mechanical Coordination of Motor Ensembles Revealed Using Engineered Artificial Myosin Filaments.**R.F. Hariadi¹, R. Sommese¹, S. Sivaramakrishnan¹;¹Cell and Developmental Biology, University of Michigan, Ann Arbor, MI

Force production and contraction in muscle are driven by interactions between actin and myosin filaments. While the individual actin-myosin cross-bridge has been extensively characterized, coordination within myosin ensembles remains poorly understood. Here, we engineer an artificial myosin filament using a polymerizable DNA nanotube scaffold that provides control over motor number and spacing. For both myosin V and myosin VI filaments, neither myosin density nor total myosin number has an effect on the gliding speed. We propose a simple model wherein myosin ensembles function as an energy reservoir that buffers individual stochastic events to provide smooth continuous motion. We find that the compliance of individual cross-bridges has been tuned to enhance mechanical coupling between motors, while minimizing Brownian effects.

P930**Board Number: B250****Dynamic actin filaments are capable of fine-tuning myosin processivity in a filament age-dependent manner.**D. Zimmermann¹, A. Janik², D.R. Kovar^{1,2}, R.S. Rock²;¹Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL, ²Biochemistry and Molecular Biology, University of Chicago, Chicago, IL

Unlike a static and immobile skeleton, the actin cytoskeleton is a highly dynamic network of filamentous actin (F-actin) polymers that continuously turn over. In addition to generating mechanical forces and sensing mechanical deformation, dynamic F-actin networks are utilized as cellular “tracks” along which specialized processively moving myosin motors transport molecular cargoes such as organelles and RNA. Unfortunately, the vast knowledge about how processive myosins function derives almost exclusively from *in vitro* studies where myosin motility was studied on pre-assembled and artificially stabilized *static* F-actin tracks. In this study we addressed the role of actin dynamics in myosin motility using a novel *in vitro* assay by which myosins traveling *dynamic* assembling F-actin can be studied over time at the single molecule level. For this, we chose the two well-characterized and highly processive motors myosin V and VI, which are heavily involved in various transport processes. We found that on dynamic F-actin the barbed end-directed motor myosin V is 1.5-fold more processive (i.e., increased runlength), whereas its pointed end-directed counterpart myosin VI is turned less processive (1.7-fold) compared to the motility behavior on static F-actin. Furthermore, our data indicate differences in myosin processivity along the length of an aging actin filament. While myosin V is more likely to take longer runs on ADP/P_i-rich (young) portions of the growing filament, myosin VI travels longer distances along ADP-rich (old) F-actin, suggesting a fine-tuning mechanism by which myosins sense the nucleotide-state of the track they are walking on. Taken together, these experiments indicate that processive myosins may gain quite different transport properties solely depending on whether a myosin travels ADP/P_i or ADP F-actin, adding a novel mechanism of how myosin traffic may sort on different F-actin networks.

P931**Board Number: B251****The mechanosensory proteome of the mammalian actin cytoskeleton.**E.S. Schiffrhauer¹, T. Luo², X. Qian², K. Mohan³, P. Iglesias³, D. Robinson^{1,4};¹Department of Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD, ²Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD, ³Electrical and Computer Engineering, Johns Hopkins University, Baltimore, MD, ⁴Department of Pharmacology and Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD

Cells have a remarkable ability to sense and respond to their mechanical environment; this ability directs a host of cellular and global processes, such as metastasis, engulfment, blood vessel constriction, and tissue development. However, the direct mechanism by which the components of the cortical

cytoskeleton assemble and interact to exert force on the membrane in response to mechanical perturbations remains unclear. The cytoskeleton is comprised of a dynamic network of actin filaments, myosin motors, and actin crosslinking proteins which generate a tunable feedback system for controlling the resting cortical tension of a cell, as well as its viscoelastic response to an added load. Here, we define the in vivo kinetics of 20 cytoskeletal and signaling proteins in response an applied external stress and identify a number of structural elements that accumulate to regions of the cell under shear or dilational forces. Prominent among the responsive proteins of the mammalian cytoskeleton are the three non-muscle myosin II paralogs, the actin bundling protein α -actinin 4, and the orthogonal actin crosslinking protein filamin B. All three myosin paralogs, as well as α -actinin 4, accumulate to the tip region of the aspirated cell, which experiences primarily dilational force. Filamin B, however, accumulates to the tip and the neck region, demonstrating that it responds to both sheared and dilated microenvironments. These differences in response can be directly related to the actin binding characteristics and distinct mechanism of force response for each protein. Interestingly, a mutation in α -actinin 4 at lysine 255, known to be the cause of the human kidney disorder Focal Segmental Glomerulosclerosis (FSGS), displays a characteristic disorganization in the actin cytoskeleton. In the proposed wild-type model, lysine 255 participates in a salt bridge that allows for a change in protein confirmation under load and a subsequent increase in the protein's actin binding affinity. This model predicts that destruction of this bridge would lead to tighter baseline binding to the actin network and, therefore, to lower turnover. Indeed, we observe much slower accumulation kinetics to the site of stress in the FSGS-associated α -actinin 4 mutant K255E. The accumulation of cytoskeletal motors and crosslinking proteins under mechanical load represents a mechanism by which cells can concentrate structural components to reduce strain or retract from a local environment of high stress. Understanding how cortical protein dynamics intrinsically give rise to the self-tuning nature of the cell will not only offer insight into the mechanisms underlying major human diseases, but also the basic nature of mechanically-dependent cell processes.

P932

Board Number: B252

An unconventional myosin mediates crosstalk between the actin and microtubule cytoskeletons.

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Plant cells divide using the phragmoplast, a microtubule-based structure that directs vesicles secretion to the nascent cell plate. The phragmoplast forms at the cell center and expands to reach a specified site at the cell periphery, tens or hundreds of microns distant. The mechanism responsible for guiding the phragmoplast remains largely unknown. Here, using both moss and tobacco, we show that myosin VIII associates with the ends of phragmoplast microtubules and together with actin guides phragmoplast expansion to the cortical division site. Our data lead to a model whereby myosin VIII links phragmoplast

microtubules to the cortical division site via actin filaments. Myosin VIII's motor activity along actin provides a molecular mechanism for steering phragmoplast expansion.

P933

Board Number: B253

AMPK-related protein kinase UNC-82/ARK5/SNARK/NUAK associates with myosin and regulates myosin organization in *Caenorhabditis elegans* striated muscle.

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In *Caenorhabditis elegans* striated muscle, the UNC-82 serine/threonine kinase is required for maintaining the organization of myosin filaments and internal components of the M-line during development. The pathology of *unc-82(0)* mutants is consistent with a failure to adapt myosin filament placement in response to embryonic cell shape change (Hoppe et al., 2010). The mechanism of UNC-82 kinase activation, the signal that promotes activation and the identities of UNC-82 targets have not been established. Antibody staining of *unc-82* mutant alleles revealed that ectopic accumulations of paramyosin occur in a kinase domain missense mutant, but not in the presumptive null. An UNC-82::GFP fusion containing the missense mutation in the kinase domain (*e1220*) shows wild-type localization in an N2 background, but localization to ectopic structures that contain paramyosin in the *unc-82* null background, suggesting paramyosin may be a substrate. The hypothesis that the kinase-impaired UNC-82 protein drives formation of these distinctive paramyosin accumulations is supported by the observation that similar paramyosin structures present in *unc-98* mutants do not form in the *unc-82*; *unc-98* double mutant. We tested for physical interaction between UNC-82 and other M-line or thick-filament proteins by using strains in which myosin, paramyosin or UNC-82 is ectopically localized within muscle cells, and determining which proteins are recruited to the ectopic structures. Our results suggest that UNC-82 physically interacts, either directly or indirectly, with one or more of these three proteins: myosin, paramyosin, and UNC-98, which is a potential chaperone required for the incorporation of paramyosin into thick filaments (Miller et al., 2008). UNC-82::GFP localized to ectopic myosin accumulations, which result from mutation of potential phosphorylation sites in the myosin nonhelical tail piece, and do not contain detectable paramyosin. Antibody staining of double mutant embryos expressing truncated myosin and homozygous for *unc-82(0)* revealed an increase in ectopic accumulations of myosin. These data suggest that UNC-82 physically associates with myosin and regulates myosin localization in a manner that does not require the myosin tailpiece. Further evidence of the role of UNC-82 in regulating myosin filament localization is further demonstrated in double mutants with the M-line kinase UNC-89/obscurin, which exhibit novel elongated myosin structures outside of the contractile apparatus. Our data are consistent with the model that misregulation of the incorporation of myosin and paramyosin into filaments is the underlying defect in *unc-82* mutants.

P934**Board Number: B254****Hat1, a lysine acetyltransferase, affects cytoplasmic mRNA storage granules.**A. Kindsfather¹, N. Rothbard¹, S. Robins¹, A. Hilliker¹;¹Biology, Univ Richmond, University of Richmond, VA

mRNAs are constantly shifting between translation and translation repression or degradation. Repressed mRNAs can accumulate with proteins in cytoplasmic foci, such as processing (P) bodies and stress granules. Ded1 is a DEAD-box RNA helicase that is essential in yeast and conserved from yeast to humans. The human homolog to Ded1, DDX3, is misregulated in several cancers and is used by both hepatitis C and HIV to promote viral life cycles.

Our previous results suggest a two-step model through which Ded1 returns repressed mRNAs to translation. First, Ded1 transitions repressed mRNAs, such as those that accumulate in P-bodies, into a pre-48S initiation complex that can accumulate in stress granules. Second, Ded1 hydrolyzes ATP and allows the mRNA to continue into translation. We hypothesize that Ded1 first recruits translation initiation factors to an mRNA and then acts as an ATP-dependent switch to allow that mRNA to recruit the ribosome. Consistent with this model, Ded1 directly binds translation initiation factors eIF4E and eIF4G. Presumably, both steps of Ded1 function are regulated to control translation of mRNAs.

In the search for regulators of Ded1, we conducted a genetic screen in order to identify factors from the yeast genome that, when overexpressed, would suppress the severe growth defect caused by *DED1* overexpression. This screen identified the histone acetyltransferase *HAT1*. We used this genetic assay to screen other lysine acetyltransferases and deacetylases for genetic interactions with *DED1*.

Hat1 shows both nuclear and cytoplasmic localization, suggesting that it could affect cytoplasmic proteins. We did not observe acetylation of Ded1 *in vivo*, as assayed by affinity purification and western blot. We also tested whether the absence or overexpression of *HAT1* affects mRNA storage granules. When visualized using fluorescence microscopy under various stress conditions, the deletion of *HAT1* increases the number and intensity of stress granules and P-bodies, suggesting that Hat1 normally either promotes the disassembly or antagonizes the formation of cytoplasmic granules. This effect on granules occurs within minutes of the stress, which is likely too short of a time span to result from nuclear changes in chromatin state and transcriptional changes. While the direct target of Hat1 acetylation is unknown, its effect on cytoplasmic granules suggests that acetylation plays a role in the regulation of cytoplasmic mRNA. These data broaden the role for a lysine acetyltransferase that is assumed to only modify histones.

P935**Board Number: B255****Investigating the Effects of Arginine Methylation on the Role of Ded1, a DEAD-Box Helicase.**N. Rothbard¹, A. Winters¹, A. Hilliker¹;¹Biology, Univ Richmond, University of Richmond, VA

Translational control is a fast and reversible way that cells respond to their environment. mRNAs can be translated, or translationally repressed and stored in cytoplasmic mRNA storage granules, such as P- (processing) bodies or stress granules. Stored mRNAs can be degraded or shuttled back into active translation. mRNA regulation can shift within minutes of a stress and the proteins that promote mRNA regulation are known to be affected by post-translational modifications.

Ded1 is an RNA helicase that has been implicated in moving translationally-repressed mRNAs into translation (1). Our previous data suggests the following two-step model: First, Ded1 promotes the movement of repressed mRNAs, such as those in P-bodies, into a pre-initiation complex that can accumulate in stress granules. Second, Ded1 hydrolyzes ATP to promote translation. Regulation of both of these functions of Ded1 is important to the regulation of mRNA translation.

Post-translational modifications represent one possible way to regulate Ded1 function. Ded1 was recently shown to be methylated at 4 arginine residues. Methylation of Ded1 increases the interaction between Ded1 and Npl3, a protein involved in mRNA export (2). We acquired mutations at one of these four arginines as an intergenic suppressor of Ded1 mutants that were impaired in the Ded1's second function in translation.

We are testing whether Ded1's arginine methylation affects Ded1's function in translation. We mutated each affected residue to lysine by site-directed mutagenesis. *DED1* is an essential gene. We are testing whether these mutant *ded1* alleles are able to complement a *ded1* null and whether these mutants confer any growth defect to yeast cells.

Subsequent experiments using these constructs will seek to determine the effects, if any, of Ded1's arginine methylation on the formation of cytoplasmic mRNA storage granules or Ded1's accumulation in stress granules. Additionally, we will test whether methylation affects Ded1's role in translation, as well as other defined biochemical roles of Ded1.

(1) Hilliker A, et al. (2011) Mol Cell. 43: 962-972.

(2) Erce M, et al. (2013) Mol Cell Proteomics. 12: 3184-3198.

P936**Board Number: B256****How is the motor force regulated in skeletal muscle contraction?.**B. Chen¹;¹Zhejiang Univ, Hangzhou, China

How the force of a molecular motor is regulated to perform the mechanical work in living systems is fascinating. Contrary to the conventional wisdom, recent experiments suggested that the motor force maintains ≈ 6 pN during skeletal muscle contraction at the steady state. Through analysis, we show that this rather precise regulation in an essentially chaotic system can be due to that a “working” motor is arrested in a transitional state. We then suggests that the motor force can be self-regulated through chemomechanical coupling. This work provides insights to understanding the coordinated function of multiple molecular motors existing in various physiological processes.

P937**Board Number: B257****Myosin Superfamily Classification using the Conserved Domain Database (CDD) Resource at NCBI.**R.A. Yamashita¹, A. Marchler-Bauer¹, S. Bryant¹;¹NIH/NCBI, Bethesda, MD

The Conserved Domain Database (CDD) at NCBI is a repository of manually curated and computationally derived protein domain families from various sources including Pfam, SMART, TIGRFAM, and COG. CDD curators use a number of tools including CDTree, CN3D, CDART, VAST, and Pubmed to create evolutionary hierarchies with detailed protein descriptions, PMIDs, annotations with graphical displays of protein-ligand interactions. We have applied these methods to create a current classification system of the ever growing myosin motor domain. We will present a comparison of our analysis with that of recent work from other researchers. A full suite of Conserved Domains and Protein Classification resources can be found here: <http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>

P938**Board Number: B258****Cellular and biochemical studies of the *Dictyostelium* alpha kinases, myosin heavy chain kinase D and alpha kinase 1.**E. Lehman¹, K. Burgett¹, A. Herring¹, P. Steimle¹;¹Biology, Univ North Carolina-Greensboro, Greensboro, NC

Contraction-dependent processes such as cytokinesis and cell migration rely on the proper assembly and localization of myosin II bipolar filaments. In *Dictyostelium* cells, as well as in mammalian cells, myosin II filament disassembly can be driven by phosphorylation of the myosin II heavy chain (MHC) "tail". MHC phosphorylation in *Dictyostelium* is catalyzed by at least three kinases (MHCK-A, -B, and -C) that share homologous alpha-kinase catalytic and WD-repeat domains. In the current study, we examined the cellular and biochemical characteristics of two other *Dictyostelium* alpha kinases - MHCK-D and Alpha kinase 1 (AK1). We report here that over-expression of MHCK-D slows suspension growth, with cells becoming large and multinucleated over time; a phenotype consistent with a role for MHCK-D in driving myosin II filament disassembly. Moreover, in vitro kinase experiments revealed that MHCK-D phosphorylates MHC to a stoichiometry of ~0.6 mol/mol MHC and in manner that leads to myosin II filament disassembly. RT-PCR analysis of expression revealed that MHCK-D, unlike the other MHCKs, is expressed only during *Dictyostelium* multicellular development (peak expression at ~16h). We also found that in live *Dictyostelium* cells GFP-tagged MHCK D exhibits robust and persistent localization to the leading edges of cellular extensions. By contrast, a truncation of MHCK-D lacking the WD-repeat domain localizes mainly to the cytoplasm, suggesting that the WD-repeat domain is involved in the targeting the kinase to cellular protrusions. Taken together, these results indicate that MHCK-D is a bone fide myosin II heavy chain kinase that is likely to function in myosin II filament turnover at the leading edges of migrating *Dictyostelium* cells.

Like MHCK-D, the AK1 protein shares sequence homology with the catalytic domains of the MHCK family, and thus may also play a role in regulating myosin II function in the cell. To explore this possibility, we have engineered a recombinant plasmid for planned studies of AK1 over-expression in *Dictyostelium* cells and for eventual affinity purification of AK1 for studies of the enzyme's catalytic activity. Collectively, our studies MHCK-D and AK1 have the potential to impact our understanding of the basic cellular functions of these two novel proteins in a variety of cellular contexts, including cell division and cell migration, both of which are impaired in cancer cells that exhibit uncontrolled multiplication and metastasis.

P939**Board Number: B259****Differential roles of myosin 1c and myosin 1b during exocytosis in alveolar type II cells.**N. Kittelberger¹, M. Breunig¹, R. Martin², H. Knoelker², P. Dietl¹, P. Miklavc¹;¹Inst. of Gen. Physiology, University of Ulm, Ulm, Germany, ²Department of chemistry, Technische Universität Dresden, Dresden, Germany

Class 1 myosins are molecular motors with actin-binding head and lipid-binding C-terminal domain, which function as linkers between actin network and membranes. Myosin 1 isoforms have different tension sensing properties and are thought to mediate different functions in the cell. We investigated the role of myosin 1c and 1b for exocytosis of surfactant-containing secretory vesicles (lamellar bodies) in primary isolated alveolar type II cells. Both myosin 1 isoforms are expressed in type II cells and localize to fused lamellar bodies in immunofluorescence experiments. Live cell imaging on cells expressing fluorescently coupled myosin constructs showed that myosin 1c and 1b have profoundly different recruitment kinetics to fused vesicles. Myosin 1c translocation closely followed PIP₂ on the vesicle membrane, whereas myosin 1b translocation was largely PIP₂ independent. Inhibition of myosin 1c using a dominant-negative tail domain significantly decreased exocytosis after ATP stimulation, which did not happen after myosin 1b inhibition. We confirmed this finding by using myosin 1c inhibitor pentachloropseudilin, which also resulted in significantly lowered exocytosis. Efficient surfactant extrusion requires formation and compression of actin coat on fused vesicles and we investigated the influence of myosin 1c and 1b on these processes. Inhibition of myosin 1c slowed down vesicle compression, whereas inhibition of myosin 1b had no such effect. In summary, myosin 1c and 1b are both present on lamellar body membrane after fusion, however myosin 1c appears to be the one with a more prominent function for secretory vesicle exocytosis in alveolar type II cells.

P940**Board Number: B260****Myosin II controls cellular branching morphogenesis and migration in 3D by responding to and minimizing cell surface curvature.**R.S. Fischer¹, H.L. Elliott², K.A. Myers³, R.A. Desai⁴, C.S. Chen^{5,6}, R.S. Adelstein⁷, C. Waterman⁸, G. Danuser⁹;

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Cell shape is integral to cell function, tissue formation, and regulation of intracellular signaling. An important program of cell shape control in neuronal, endothelial, and cancer cells is branching morphogenesis, in which migratory cells invade a 3D extracellular matrix (ECM) by developing a complex arboreal morphology with a spindle-shaped cell body and branch-like protrusions extending into the ECM. While it has been shown that actin polymerization can drive branch formation and that myosin II inhibits branching, three central questions remain unresolved regarding the control of 3D cell shape by actomyosin. First, how is the molecular-scale activity of myosin II motors related to the cell-scale shape? Second, does cell shape feed back to regulate actomyosin? And third, how is actomyosin spatially and temporally controlled to mediate branching and guide migration? We utilize high-resolution imaging, computer vision, and 3D mathematical morphology measurements to quantify branching morphogenesis and migration in primary endothelial cells in 3D collagen ECMs. We find that myosin-II motor activity regulates micro-scale cell surface curvature to control cell-scale branch complexity and orientation. Myosin-II preferentially assembles onto cortical regions of minimal surface curvature while also acting to minimize local curvature. Using micropatterned chambers, we demonstrate that myosin II assembly is biased by local cortical curvature. We also find that curvature-dependent cortical assembly of myosin II could be separated from its ability to generate tension by inhibiting myosin II force generation with either blebbistatin or a mutant myosin II heavy chain (R702C) with impaired motor function. Using automated 3D curvature mapping and branch analysis of live cell migration in 3D demonstrates that cycles of exploratory branching followed by branch pruning and cell body advance are driven by cycles of myosin-II dissociation from the cortex that allow plasma membrane curvature to increase, followed by myosin-II cortical recruitment to drive curvature minimization. Differential association of myosin to outer low-curvature and inner high-curvature surfaces of branches controls branch orientation, linking local curvature control to global directional control of migration. Thus, cell surface curvature minimization is a central mechanism that translates the molecular activity of myosin-II at the cortex into dynamic shape control for guiding cell migration in 3D.

Kinesins 1

P941

Board Number: B261

Why is Kinesin-2 KIF3AC so Processive?.

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Mammalian KIF3AC is a heterotrimeric kinesin-2 that consists of two non-identical N-terminal motor polypeptides that dimerize and bind a nonmotor kinesin-associated-protein (KAP) to form the C-terminal cargo-binding domain. KIF3AC is best known for its roles in neuronal tissue for organelle transport and remodeling of the microtubule cytoskeleton, yet *in vitro* studies to characterize its single molecule behavior are lacking. We report that kinesin-2 KIF3AC is highly processive with run lengths at $1.23 \pm 0.09 \mu\text{m}$ which match those exhibited by conventional kinesin K560 at $1.26 \pm 0.1 \mu\text{m}$ in comparative experiments. This result was unexpected because KIF3AC exhibits the canonical kinesin-2 neck linker sequence that has been reported to be responsible for the shorter run lengths observed for kinesin-2 KIF3AB. KIF3C also contains a long extension in surface loop L11 not present in KIF3A or B, or other processive kinesins. Loop L11 is a component of the kinesin microtubule interface and has been implicated in activation of ADP release when kinesin collides with the microtubule. KIF3AC encoding a truncation in KIF3C L11 (KIF3AC Δ L11) is even more processive than wildtype KIF3AC at $1.55 \pm 0.09 \mu\text{m}$. This observation suggests that loop L11 also plays a role in determining the run length. Steady-state ATPase experiments show that shortening L11 does not alter the k_{cat} , consistent with the observation that single molecule velocities are not affected by shortening loop L11. However, shortening loop L11 does significantly increase microtubule affinity: $K_{1/2,MT} = 232.8 \pm 26.6 \text{ nM}$ for KIF3AC and $169.3 \pm 14.4 \text{ nM}$ for KIF3AC Δ L11. Analysis of the homodimers KIF3CC and KIF3CC Δ L11 reveals that both are processive but exceedingly slow. The run length of KIF3CC at $0.57 \pm 0.03 \mu\text{m}$ is shorter than that of KIF3CC Δ L11 at $0.75 \pm 0.03 \mu\text{m}$, and the steady-state ATPase $K_{1/2,MT}$ for KIF3CC at $44.3 \pm 7.4 \text{ nM}$ is weaker than the $K_{1/2,MT}$ for KIF3CC Δ L11 at $8.4 \pm 0.2 \text{ nM}$. These results reveal that processivity can be regulated in part by loop L11 length, consistent with the interpretation that loop L11 becomes ordered upon microtubule collision to activate ADP release. These studies also expand our understanding of motor processivity and point to alternative structural and mechanistic modulators of processivity. Supported by NIH grants to I. Rayment (R01-GM086351) and S. P. Gilbert (R37-GM54141). K. C. Rank was supported by NIH training grant (T32-GM07215).

P942**Board Number: B262****Cargo-mediated dimerization of mammalian Kinesin-3 motors results in superprocessive motion.**V. Soppina¹, S. Norris², A.S. Dizajia¹, M. Kortusa¹, S.L. Veatch², M. Peckham³, K.J. Verhey⁴;¹Cell and Developmental Biology, University of Michigan, Ann Arbor, MI, ²Biophysics, University of Michigan, Ann Arbor, MI, ³Univ Leeds, Leeds, England, ⁴Department of Cell and Developmental Biology, University of Michigan Medical School, Ann Arbor, MI

The kinesin-3 family is one of the largest among the kinesin superfamily and its members play important roles in a wide range of cellular transport activities, yet the molecular mechanisms of kinesin-3 regulation and cargo transport are largely unknown. We performed a comprehensive analysis of mammalian kinesin-3 motors from three different subfamilies (KIF1, KIF13, and KIF16). Using Forster resonance energy transfer microscopy in live cells, we show for the first time to our knowledge that KIF16B motors undergo cargo-mediated dimerization. The molecular mechanisms that regulate the monomer-to-dimer transition center around the neck coil (NC) segment and its ability to undergo intramolecular interactions in the monomer state versus intermolecular interactions in the dimer state. Regulation of NC dimerization is unique to the kinesin-3 family and in the case of KIF13A and KIF13B requires the release of a proline-induced kink between the NC and subsequent coiled-coil 1 segments. We show that dimerization of kinesin-3 motors results in superprocessive motion, with average run lengths of $\approx 10 \mu\text{m}$, and that this property is intrinsic to the dimeric kinesin-3 motor domain. This finding opens up studies on the mechanistic basis of motor processivity. Such high processivity has not been observed for any other motor protein and suggests that kinesin-3 motors are evolutionarily adapted to serve as the marathon runners of the cellular world.

P943**Board Number: B263****A novel system for assembling multi-kinesin complexes in live cells reveals independent action of kinesins in complex.**S. Norris¹, V. Soppina², D. Cai², S. Sivaramakrishnan², K.J. Verhey³;¹Biophysics, University of Michigan, Ann Arbor, MI, ²Cell and Developmental Biology, University of Michigan, Ann Arbor, MI, ³Department of Cell and Developmental Biology, University of Michigan Medical School, Ann Arbor, MI

Processive molecular motors such as kinesin and dynein are largely responsible for the trafficking and organization of subcellular cargoes, including organelles, vesicles, mRNA particles, and even viruses, in eukaryotic cells. Although the biophysical and biochemical properties of individual motor proteins are well-characterized, how motors coordinate their motility when attached to the same cargo is largely unknown. To study the emergent properties of multi-motor complexes, we developed a novel protein-

based system to assemble defined complexes of kinesin motors inside living cells. We first characterized protein segments that can serve as a scaffold for assembling complexes or as linkers for attaching kinesin motors to the scaffold. We next tested the effectiveness of ten different fluorescent proteins for two-color imaging of kinesins in complex and found that EGFP, monomeric EGFP, tagRFPT, and mApple resulted in a population of motors with aberrant run length behavior. Finally, we applied the system to study the cooperation between slow kinesin-1 motors and fast kinesin-3 motors attached to the same complex. We find that the motors act independently (neither hinder nor help each other), both in vitro in cell lysates and in live COS7 cells, and that the motors can alternate their actions. We find that slow kinesin-1 motors dominate the motility of the complex in vitro on homogeneous microtubules but that kinesin-3 can dominate in live COS7 cells where microtubule heterogeneity provides tracks that kinesin-1 does not engage with. We find that motors can merge with and separate from the scaffold during motility events, suggesting a force-dependent release of motor-cargo linkages.

P944

Board Number: B264

Kinesin-5 acts as a microtubule stabilizer, polymerase and plus-tip tracker.

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Kinesin-5 slides antiparallel microtubules apart during mitosis and is necessary for bipolar spindle formation. Besides its unique homotetrameric configuration, determined by the coiled-coil domain, kinesin-5 motor domains also possess specific properties optimal for their spindle organizing function. To study properties intrinsic to the kinesin-5 motor domain, we generated functional kinesin-5 dimers by fusing the kinesin-5 head and 18-residue neck linker to the coiled-coil rod of kinesin-1. In ATP this kinesin-5 dimer decorated plus-ends of taxol-stabilized microtubules and slowed depolymerization of GMPCPP microtubules. On dynamic microtubules, kinesin-5 dimer increased the microtubule growth rate by more than a factor of two and reduced the catastrophe frequency three-fold. These findings suggest kinesin-5 can stabilize microtubules and acts as a microtubule polymerase. To understand this polymerase mechanism, TIRF microscopy was used to visualize individual GFP-labeled kinesin-5 dimers on immobilized microtubules. Motors walked to the ends of microtubules and remained bound there for 7 seconds, much longer than the 0.1 s motor step time. A more processive kinesin-5 dimer, made by shortening the neck linker to 14-residues, tracked the plus-ends of growing microtubules and had similar end-binding durations. These data suggest that after walking to microtubule plus-ends, kinesin-5 waits there instead of dissociating, and will resume walking if additional tubulin dimers are incorporated. We hypothesize that kinesin-5 promotes microtubule polymerization by stabilizing longitudinal interactions between tubulin subunits on a single protofilament. Consistent with this protofilament stabilization hypothesis, fluorescence analysis suggested that microtubule plus-ends are more tapered in the presence of kinesin-5. Furthermore, curved and looped ends were observed, which occasionally

resolved into straight microtubules, consistent with kinesin-5 stabilizing long protofilament bundles. These findings suggest that when sliding apart antiparallel microtubules in the mitotic spindle, kinesin-5 pauses at and promotes microtubule polymerization of dynamic microtubule plus-ends. Both of these activities enhance the ability of kinesin-5 to establish and maintain spindle pole separation during mitosis.

P945

Board Number: B265

Solution of the complete kinesin-2 hydrolysis cycle provides insight into bidirectional transport.

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The kinesin-2 family motor KIF3A/B coordinates with dynein in the bidirectional transport to deliver intraflagellar particles, melanosomes, neuronal vesicles, and mitotic spindle apparatus. In stark contrast to the conventional kinesin, the run length of kinesin-2 shows stronger force sensitivity, suggesting that the function of kinesin-2 is specifically adapted for bidirectional transport. To interpret the physiological behavior of kinesin-2 from a microscopic aspect, we investigated the kinetics of a functional homodimer of mouse KIF3A that exhibits similar motility to full-length KIF3A/B. By referring to the scheme developed for kinesin-1, we solved a hydrolysis model of the kinesin-2 with our kinetic assays, which are in agreement with the precedent measurements in velocity, run length, randomness, and microtubule affinity assay. This study provides direct evidence in that kinesin-2 resides longer in weak-binding state (~50%), which explains its higher force sensitivity.

P946

Board Number: B266

Kinesin-12 Kif15 targets kinetochore-fibers via an intrinsic two-step mechanism.

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Proteins that recognize and act on specific subsets of microtubules (MTs) enable the varied functions of the MT cytoskeleton. We recently discovered that Kif15 localizes exclusively to kinetochore-fibers (K-fibers), or bundles of kinetochore-MTs within the mitotic spindle. It is currently speculated that the MT-

associated protein TPX2 loads Kif15 onto spindle MTs, but this model has not been rigorously tested. Here, we show that Kif15 accumulates on MT bundles as a consequence of two salient biochemical properties. First, Kif15 is self-repressed by its C-terminus. Second, Kif15 harbors a non-motor MT-binding site, enabling dimeric Kif15 to crosslink and slide MTs. We further show that two-MT-binding activates Kif15, resulting in its accumulation on and motility within MT bundles but not on individual MTs. We propose that Kif15 targets K-fibers via an intrinsic two-step mechanism involving molecular unfolding and two-MT-binding. This work challenges the current model of Kif15 regulation and provides the first account of a kinesin that specifically recognizes a higher order MT array.

P947

Board Number: B267

Src Phosphorylation Regulates the Human Kinesin 5, Eg5, and Disrupts the Binding of Eg5 Inhibitors.

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Eg5 is a human kinesin-5 that drives spindle pole separation during the early phases of mitosis. While a substantial body of work has revealed the role that Eg5 plays during mitosis, relatively little is known about how Eg5 activity is regulated throughout the cell cycle. Our data shows that endogenous Eg5 in HEK cells is phosphorylated on tyrosine residues. *In silico* predictors suggest three tyrosines in the Eg5 motor domain as targets of Src kinase phosphorylation. We show that these residues are phosphorylated specifically by Src kinase, but not by Wee1 kinase *in vitro*. Furthermore, cells expressing Eg5 constructs with a phosphomimetic mutation of Y211 to glutamate (Y211E) show significantly higher percentages of monopolar spindles than cells expressing wild-type Eg5. The Y211E mutation also significantly decreases Eg5 ATPase rates and motility *in vitro*. The proximity of the potential phosphorylation sites to the binding sites of small molecule inhibitors targeting Eg5 suggested that phosphorylation may interfere with drug binding. Isothermal calorimetry experiments show that phosphomimetic Eg5 constructs have significantly decreased affinity for S-trityl-L-cysteine (STLC). Together, these data suggest a role for the mitotic kinase Src in regulating Eg5 throughout the cell cycle by directly altering its motor characteristics. Such a role for post-translational modifications has not yet been investigated in Eg5. Additionally, the effect of phosphomimetic mutations on STLC binding suggests that phosphorylation may decrease the efficacy of Eg5 inhibitors that are currently in clinical trials. Future work will seek to confirm Src kinase as a regulator of Eg5 activity in cells and investigate phosphorylation as a potential cancer resistance mechanism.

P948**Board Number: B268****The C-terminal region of MCAK controls structure and activity through a conformational switch.**J.P. Welburn¹, S. Talapatra¹, B. Harker¹, A. Bertin²;¹Wellcome Trust Centre for Cell Biology, University of Edinburgh, Edinburgh, Scotland, ²Inst Curie- Umr CNRS 168, Paris, France

Motor proteins in the Kinesin-13 family are potent microtubule depolymerases that trigger microtubule catastrophe. The non-motor regions flanking the motor domain regulate Kinesin-13 function through phosphorylation. However the molecular basis for the regulation of their activity is not well understood. Here using X-ray crystallography and electron microscopy, we report that the kinesin-13 MCAK forms a compact structure in solution, unlike canonical kinesins. This compact structure of MCAK is held auto-inhibited by an interaction with its C-terminus to prevent non-productive ATP hydrolysis and reduce the affinity of MCAK for microtubules in vitro. The C-terminus of MCAK promotes motor head dimerization, favoring a double lockdown inhibitory mechanism, similarly to Kinesin-1 inhibition. The X-ray structure of MCAK bound to the C-terminus at 3Å reveals that the binding of the ATPase domains as dimers, with the C-terminus binding at the interface between the two ATPase domains. Finally, we show that phosphorylation of the C-terminus of MCAK alleviates MCAK auto-inhibition and induces a large conformational rearrangement. We propose that this regulatory mechanism allows phosphorylation-dependent spatial and temporal activation of MCAK depolymerase activity during mitosis.

P949**Board Number: B269****Sensitivity of Multiple-Kinesin Transport to Microtubule Lattice Defects.**J. Xu¹;¹Univ California-Merced, Merced, CA

Microtubules are fundamental biopolymers in cells, formed via self-assembly of tubulin dimers. Defects in microtubule lattices have been observed, including point defects (missing tubulin dimers) and line defects (protofilament disruption). Microtubule-based molecular motors enable long-range transport in cells. Potential impact of microtubule lattice defects on intracellular transport is not yet understood. Here we vary microtubule polymerization conditions to uncontrollably tune defect presence in microtubule lattices, and use single-molecule-type optical trapping experiments to investigate the impact of such defect on multiple-kinesin transport. We find that kinesin-based cargoes pause preferentially at specific locations along individual microtubules investigated, and that the pause frequency and duration increase with increasing presence of defects in microtubules. Additionally, we find that the dissociation rates of multiple-kinesin-based cargoes are also strongly dependent on the specific microtubules they travel along. Taken together, our study highlights a previously unexplored, and important role of microtubule polymerization condition in regulating intracellular transport.

P950**Board Number: B270****Loss of fission yeast kinesin-14 pkl1p results in aneuploidy via chromosome cut.**V. Syrovatkina¹, P. Tran^{1,2};¹Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA, ²CNRS UMR144, Institut Curie, Paris, France

Fidelity of chromosome segregation requires the proper formation of the bipolar mitotic spindle. The spindle – organized by microtubules, motors, and microtubule-associated proteins – consists of the spindle poles which nucleate microtubules and focus the microtubule minus ends, the interpolar microtubules which form the spindle midzone containing crosslinked antiparallel overlapping microtubule plus ends, and the kinetochore microtubules which make kinetochore-microtubule attachment for subsequent chromosome segregation. Defects in spindle pole microtubule focusing have led to defects in chromosome segregation, resulting in aneuploidy, a hallmark of cancer cells. However, the mechanism of how unfocused spindle poles lead to aneuploidy is not well understood. Here we show that fission yeast kinesin-14 pkl1p focuses the minus ends of microtubules at the poles during mitosis. The absence of pkl1p (pkl1Δ) leads to fragmentation of the spindle poles, unfocused microtubule minus ends, and aberrant microtubule protrusions away from the spindle axis. The microtubule protrusions are produced by kinesin-5 cut7p, located at the spindle midzone, sliding microtubules apart. Surprisingly, chromosome segregation proceeds normally during mitosis in pkl1Δ cells. However, at the end of mitosis, some long minus end microtubule protrusions can reach the cell tip cortex and push the properly segregated chromosome mass toward the medial cell division site, where the contractile ring can “cut” the chromosome and produce aneuploidy. This novel pathway for aneuploidy may be particularly dangerous, as it bypasses the known checkpoints that ensure chromosome segregation fidelity.

P951**Board Number: B271****Defective axonal transport increases Aβ production downstream from kinesin-1-assisted phosphorylation of amyloid-β precursor protein by JNK.**V. Muresan¹, Z. Ladescu Muresan¹;¹Pharmacology and Physiology, New Jersey Medical School - Rutgers University, Newark, NJ

Stress transiently impairs normal cellular function; prolonged, or repeated stress episodes, occurring throughout life, could lead to cancer or neurodegenerative diseases, including Alzheimer's disease (AD). Here we show that, in neurons, stress impairs axonal transport, which triggers a signaling cascade that delays the folding, and the export from the endoplasmic reticulum (ER), of the Amyloid-β Precursor Protein (APP), a transmembrane protein relevant to AD. We find that, normally, APP is transported from the soma to the neurites by recruiting kinesin-1 via the adaptor protein Fe65, an interactor of APP, and

of the cargo binding subunit of kinesin-1, the KLC. A fraction of APP is proteolytically cleaved into fragments, including the amyloid- β peptide (A β); in AD, the increased generation and accumulation of A β oligomers is neurotoxic. APP is phosphorylated by c-Jun NH2-terminal kinase (JNK) at Thr668, an event that increases its susceptibility to amyloidogenic cleavage. With cultured neurons, we find that the phosphorylation of APP is enabled by kinesin-1, which mediates - via its two KLCs - recruitment of JNK to APP. We show that APP phosphorylation requires the divalent KLCs, which provide the platform for recruiting JNK - via JIP-3 (a JNK scaffold) - and enable its anchoring to APP - via Fe65. Silencing the expression of Fe65, JIP-3, or KLC reduces, whereas the moderate overexpression of any of them increases, the levels of phosphorylated APP. We find that the kinesin-1-assisted phosphorylation of APP does not require the motor activity of kinesin-1, or its binding to microtubules. By contrast, APP phosphorylation is enhanced in conditions of impeded axonal transport, when APP, Fe65, kinesin-1, JIP-3, and JNK are retained in the soma, and form a complex at the ER/ER-Golgi-intermediate compartment (ERGIC). We also find that the phosphorylated APP that is generated in the soma upon blocking of transport is rapidly cleaved by secretases via the amyloidogenic pathway, with subsequent accumulation and oligomerization of A β in the ER lumen, a situation that - we show - reproduces the intraneuronal accumulation of A β in the AD brain. Finally, we show that the above-described mechanism for the generation of A β , following phosphorylation of APP at the ER, is a direct consequence of impeded axonal transport, caused by a variety of forms of stress: genotoxic, osmotic, oxidative, or metabolic. Overall, this study reveals a novel, motor-independent role of kinesin-1 in cargo phosphorylation. It also uncovers an ER stress mechanism leading to the overproduction of A β in response to impeded axonal transport, which could lead to the development of AD. Supported by NIH award AG039668 (Z.M.) and by New Jersey Health Foundation awards (ZM, VM).

P952

Board Number: B272

Genetically engineered kinesin motors amenable to selective small molecule inhibition.

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The human genome codes for 45 kinesins that contain a highly conserved kinesin motor domain and divergent tail domains for their specific functions. Genetic methods such as RNAi and overexpression of dominant negative species have been used to explore the cellular roles of different kinesins. These methods have yielded valuable information, but are slow acting and prone to off-target effects and hence not suitable to address complex and dynamic biological questions. The identification of small molecule inhibitors specific to different kinesins is highly desirable, yet only a handful of such inhibitors

have been identified due at least in part to the high conservation of the kinesin motor domain. Here we demonstrate that the kinesin motor domain can be engineered to maintain motility yet be inhibited by small, cell permeable molecules. Using kinesin-1 as a prototype, we pursued two strategies to obtain inhibitable motors: I) We inserted the six amino acid tetracysteine tag into surface loops of the motor domain such that binding of biarsenic dyes conformationally distorts and thereby inhibits motility. II) We fused DmrB dimerization domains to the motor heads such that addition of B/B homodimerizer cross-links the motor domains and inhibits motor stepping. We show using cellular assays that the engineered kinesin-1 motors are able to transport artificial cargoes similarly to the wild type motor, but are efficiently inhibited by the addition of inhibitor. *In vitro* assays revealed that inhibitor addition reduces the number of active motors on the microtubule, with minor effects on motor run length and velocity. Future studies will enable us to deploy the inhibitable kinesin-1 motors in cells and animals to study cellular kinesin function with high specificity and temporal resolution. It is likely that these inhibition strategies can be successfully applied to other members of the kinesin superfamily, owing to the high conservation of the kinesin motor domain. MFE was supported by an early postdoc mobility fellowship (PBZHP3_141433) from the Swiss National Science Foundation.

P953

Board Number: B273

Antioxidant signaling involving the microtubule motor KIF12 is an intracellular target of nutrition excess in beta cells.

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Beta cell injury due to oxidative stress is a typical etiology of diabetes caused by nutritional excess, but its precise mechanism remains largely elusive. Here, we demonstrate that the microtubule motor KIF12 mediates a novel antioxidant cascade in beta cells as an intracellular target of excess fat intake or 'lipotoxicity'. Newly generated KIF12-knockout mice suffer from hypoinsulinemic glucose intolerance due to increased beta cell oxidative stress. Using this model, we identified an antioxidant signaling cascade involving KIF12 as a scaffold for the transcription factor Sp1. The stabilization of nascent Sp1 appeared to be essential for proper peroxisomal function by enhancing Hsc70 expression, and the pharmacological induction of Hsc70 expression with teprenone counteracted the oxidative stress. Because KIF12 is transcriptionally downregulated by chronic exposure to fatty acids, this newly identified antioxidant cascade involving KIF12 and Hsc70 is proposed to be a critical target of nutritional excess in beta cells in diabetes.

P954**Board Number: B274****Regulation of the kinesin Kif15 by the microtubule associated protein TPX2.**S. Balchand¹, B.J. Mann², H.M. Jordan³, P. Wadsworth²;¹Department of Biology, University of Massachusetts, Amherst, Amherst, MA, ²Department of Biology, Molecular and Cellular Biology Graduate Program, University of Massachusetts Amherst, Amherst, MA,³University of Massachusetts Amherst, Amherst, MA

The proper assembly of a bipolar mitotic spindle is essential for the faithful segregation of chromosomes. The plus-end directed motor Eg5 is known to be required in many organisms for formation of a bipolar spindle. Recently, another plus-end directed motor, Kif15, was shown to be capable of compensating for loss of Eg5 function under certain conditions. It has also been shown that Kif15 stabilizes the spindle microtubules in metaphase and makes the spindle resistant to STLC treatment. Interestingly, *in vitro* experiments have shown that the microtubule associated protein TPX2 reduces the stepping of both Eg5 and Kif15 on microtubules thereby regulating them. However, the requirements for Kif15 binding to microtubules and its regulation in cells are yet to be completely understood. So, we set out to examine whether the microtubule binding protein TPX2 regulates the function of Kif15 in cells. We observe that fluorescently tagged Kif15 remains cytosolic in interphase and localizes to spindle microtubules in mitosis. GFP-Kif15 cells are sensitive to STLC when treated for short times and become resistant to STLC following longer treatments. Resistance to STLC was not dependent on the presence of K-fibers presumably because of the overexpression of Kif15. But, when TPX2 is depleted using siRNA, GFP-Kif15 cells regain sensitivity to STLC treatment. Expressing truncated constructs of TPX2 lacking some residues in the C terminus does not rescue the functionality of Kif15 in these cells. In microtubule pelleting assays, the C terminal tail fragment of Kif15 pellets with the microtubules only in the presence of TPX2. Together these results demonstrate that TPX2 is required for the localization and function of Kif15 *in vivo*, and that residues in the C-terminal region of TPX2 are necessary for Kif15 function.

Microtubule Dynamics and Its Regulation

P955

Board Number: B276

Generating the chemical complexity of tubulin: insights into the mechanism of tubulin post-translational modification enzymes.

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Microtubules are dynamic polymers that grow through the GTP dependent addition of tubulin dimers. Cells constantly adjust the balance between dynamic, short-lived and stable, long-lived microtubules. Microtubules are decorated with abundant, chemically diverse and evolutionarily conserved posttranslational modifications that serve to mark subpopulations of microtubules for specialized functions in cells. Other than viruses and clathrin cages, microtubules are the only known hollow polymers in eukaryotic cells. Acetylation on α -tubulin Lys40 stands out as the only known tubulin posttranslational modification located in the microtubule lumen. It marks stable, long-lived microtubules and is required for polarity establishment and directional migration. Tubulin acetyltransferase (TAT) presents a puzzle: its activity is stimulated by microtubules, yet the luminal location of Lys40 hinders access by the enzyme. TAT was proposed to access its substrate by diffusing from microtubule ends, accessing the Lys40 loop from the exterior of the microtubule or by gaining access to the lumen through openings or defects in the microtubule lattice. To elucidate substrate access by TAT and to uncover the molecular basis for the *in vivo* correlation between acetylation and microtubule age, we combined X-ray crystallography, electron microscopy, biochemical and single-molecule fluorescence analyses, and modeling. We report the first crystal structure of TAT in complex with a tubulin peptide–ac-CoA bisubstrate analog that together with structure-based functional analyses constrains the active enzyme to the microtubule lumen and reveal the structural determinants for Lys40 loop recognition. Unexpectedly, we find that acetylation by TAT proceeds stochastically along the microtubule without a preference for ends despite the luminal location of the acetylation site. Consistent with this, single-molecule fluorescence imaging reveals that TAT efficiently and stochastically scans the microtubule, while modeling and kinetic analyses demonstrate that TAT catalytic activity, rather than diffusion through the lumen, is rate-limiting. Consistent with this, our high-resolution 1.35Å X-ray complex crystal structure reveals that the TAT active site is suboptimal for supporting the proton transfer required for acetylation. Thus, by virtue of its preference for the microtubule geometry and its low catalytic rate, TAT alone can function as an enzymatic timer for microtubule lifetimes.

P956**Board Number: B277****Combinatorial Regulation of Microtubule Dynamics by Microtubule Associated Proteins.**

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The lengths of spindle and astral microtubules are tightly regulated for proper eukaryotic cell division. Microtubule associated proteins (MAPs) control microtubule dynamics to tune microtubule length distributions. The roles that individual MAPs play in the regulation of microtubule dynamics have been successfully characterized using *in vitro* reconstitution. However, how combinations of MAPs work together to regulate microtubule length is often poorly understood.

During the 2014 MBL Physiology course in Woods Hole, we reconstituted the interactions of three sets of MAPs that co-localize on microtubules. We studied the effect of the budding yeast plus-end tracking protein complex Kip2p-Bik1p-Bim1p on microtubule dynamics. Both Kip2p and Bim1p promoted growth by increasing the microtubule growth rate. Addition of Bim1p, Bik1p or both to Kip2p did not further enhance microtubule growth rate or microtubule length. Furthermore, we reconstituted the competition between the budding yeast polymerase Kip2p and the depolymerase Kip3p. At equimolar concentrations of these proteins, microtubules were longer than in the absence of Kip2p and Kip3p, indicating that Kip2p had won. This result qualitatively agrees with a random walk model of microtubule dynamics and suggests that the growth promoting activity of Kip2p is dominant over the depolymerase activity of Kip3p. Finally, we examined the role of *Xenopus laevis* TPX2 and XMAP215 on microtubule nucleation. Both TPX2 and XMAP215 individually increased the number of nucleation events and TPX2 alone decreased the lag time between microtubule catastrophe and growth. Combined however, TPX2 and XMAP215 additively increased the number of nucleation events and decreased the lag time between growth events.

These studies show that the interplay of MAPs on microtubules can give rise to differences in microtubule length control beyond the effects of the individual components.

P957**Board Number: B278****A combined cell biological and biophysical strategy to analyze actin/microtubule crosstalk and its relation to cytoplasmic streaming.**M. Drechsler¹, R. Goldstein², I.M. Palacios¹;¹Department of Zoology, University of Cambridge, Cambridge, United Kingdom, ²DAMTP, University of Cambridge, Cambridge, United Kingdom

The actin and microtubule (MT) cytoskeletons constitute versatile and highly dynamic protein networks regulating a broad variety of processes like cargo transport or cellular motility. While there is a good understanding of the roles of either type of filament, knowledge about how actin and MTs interact remains rather elusive. Therefore, understanding fundamental concepts of cytoskeletal crosstalk constitutes an important task in cell biology.

We use the *Drosophila* oocyte as model of cytoskeletal crosstalk and dynamics. One major function of these two cytoskeletons in the oocyte is the asymmetric distribution of key developmental determinants. In addition to cargo transport, MTs and the motor Kinesin-1 (Kin) are essential for the induction of major bulk movements of the cytoplasm – a process known as cytoplasmic streaming. We applied particle image velocimetry (PIV) to monitor and quantify cellular fluid dynamics in living oocytes. With this technique we could detect even subtle changes in Kinesin activity and found that flow features strongly correlate with the architecture of the MT cytoskeleton^[1]. We now aim to understand how flows feedback on the MT network and how the cell maintains cytoskeletal organization, by simultaneously monitoring MT dynamics and flows in living oocytes, and subsequent analysis by PIV.

Additionally to Kin and MTs, streaming and cargo localization is regulated by a cytoplasmic F-actin mesh through a yet unknown mechanism. The formation of this actin mesh is developmentally regulated. When it is present, oocytes display slow unorganized cytoplasmic flows, while streaming becomes fast and directed when the mesh dissolves later on. The major task is now to understand how exactly the actin mesh influences Kin activity and the organization of the MT network, as well as the biophysical properties of the cytoplasm. In particular we concentrate on the function of actin-MT crosslinking proteins that might be involved in the interaction of both cytoskeletons. Preliminary data, obtained from live microscopy, suggest that the absence of flows causes an ectopic correlation between the actin mesh and MT bundles, indicating an association of both filament types.

The characterization of flows by PIV, in combination with live video microscopy, FRAP and photoconversion approaches constitutes a novel straight forward experimental setup to gain mechanistic insights into the crosstalk between F-actin and MTs in relation to cytoplasmic flows.

^[1] Ganguly et al. (2012) "Cytoplasmic streaming in *Drosophila* oocytes varies with kinesin activity and correlates with the microtubule cytoskeleton architecture" PNAS 18;109(38)13109-14

P958**Board Number: B279****A novel microfluidics assisted assay for microtubule dynamics directly links cap transitions and stability.**C. Duellberg¹, N.I. Cade², T. Surrey², D. Holmes³;¹Microtubule Cytoskeleton Laboratory, London Research Institute, London, United Kingdom, ²Cancer Research UK London Research Institute, London, United Kingdom, ³London Centre for Nanotechnology, University College London, London, United Kingdom

Switching between growth and shrinkage is an important property of microtubule ends. The molecular mechanism underlying this switch is still not well understood. Using a novel microfluidics-assisted total internal reflection fluorescence microscopy assay, we directly tested predictions of the most prominent current models of dynamic instability. We found that the mechanism of catastrophe induction cannot be explained satisfactorily by recently proposed models. We combined our microfluidics assay with novel tools to manipulate GTPase cycle-associated transition kinetics. Faster kinetics directly accelerated the disappearance of the stabilizing end structure at microtubule ends after microfluidics-assisted tubulin removal. This is the first and most direct experimental demonstration of how the lifetime of a transiently existing stabilizing end structure determines overall microtubule stability. We further show that a conformational cap recognized by EB proteins is (or at least is the major part of) the stabilizing end structure. This allowed us to directly visualize the time course of cap decay on the single filament level after microfluidics assisted tubulin removal. Cap density and lifetime - rather than total cap length - are most crucial for microtubule stability. Fluctuations in cap density likely explain microtubule lifetime distributions at steady state and after rapid tubulin wash-out. Our results provide a simple, yet powerful conceptual framework that explains the molecular mechanism of catastrophe induction.

P959**Board Number: B280****p150Glued-interacting domains of TRAPPC9 reveal regulatory function of TRAPPC9 at the centrosome.**W. Gan¹, M. Zong¹, A. Satoh², H. Chan³, S. Yu¹;¹Chinese University of Hong Kong, Hong Kong, Hong Kong, ²Okayama Univ, Okayama, Japan, ³Epithelial Cell Biology Research Center, School of Biomedical Sciences, Faculty of Medicine, The Chinese University of Hong Kong, Hong Kong, Hong Kong

The vesicle tethering factor TRAPP (Transport protein particle) complex has been implicated in cellular processes other than vesicle trafficking. Increasing evidence suggests that TRAPP has microtubule-related functions. We have recently discovered TRAPPC9, a subunit of TRAPPII, interacts with CTGlued, the cargo-binding, carboxyl terminal domain of p150Glued, a subunit of dynactin. Here, we identify three TRAPPC9 domains that mediate this interaction - p150Glued-interacting domains 1, 2 and 3 (GID1,

2 3). Purified recombinant GID1 and GID3 could interact with CTGlued in vitro, demonstrating a direct, physical interaction. Pre-incubation of GID3 with CTGlued competitively reduced the interaction between CTGlued and GID1, suggesting GID1 and GID3 bind to CTGlued at the same site. Unexpectedly, GFP-GID1 and GFP-GID3 targeted to the centrosome and affects the formation of microtubule bundles originating from the organelle. Endogenous TRAPPC9 signal also targeted to the centrosome when its Golgi-localization signal was removed by the detergent digitonin. In human skin fibroblast isolated from an individual with mental retardation and microcephaly, mutant TRAPPC9 caused increased number of centrosomes, and thus, higher chromosome ploidy level. Taken together, our results imply that TRAPPC9 plays important roles in microtubule-based axonemes, in addition to its well-documented function in vesicle traffic.

P960

Board Number: B281

Role of molecular motors and microtubule-binding proteins in cell polarity and regeneration of *Stentor*.

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Every living cell performs a multitude of operations during its cell cycle. These may include attachment to substrate, movement, division, etc. All of these processes are affected by physical properties of the cells and by their capacity to generate force. Forces are mostly generated within cells via the action of molecular motors pushing and pulling on diverse cytoskeletal structures. The unicellular ciliate *Stentor* is an excellent model system for studying force generation due to its giant size, wide range of behaviors, extraordinary level of structural regularity of its cytoskeleton, and amenability to microsurgical manipulation. Importantly, *Stentor* has the capacity to regenerate any of its parts, which gives access to studying the development of force generation structures over time and the role of these structures in regeneration. In this study, we used RNAi to analyze the role of *Stentor* cytoskeleton and molecular motors in cell motility, cell shape control, intracellular transport, and regeneration. We showed that proteins controlling microtubule dynamics have dramatic effects both on the morphology of *Stentor* cells and on their dynamic behaviors.

P961**Board Number: B282****Dynein-generated forces bend and reorient microtubules by acting upon stationary pinning points.**I.A. Kent¹, P. Rane², A.J. Ladd², T.P. Lele¹, R.B. Dickinson¹;¹Chemical Engineering, University of Florida, Gainesville, FL, ²Chemical Engineering, Univ Florida, Gainesville, FL

Microtubules dynamically bend in living cells, but the mechanisms by which microtubule shape is determined are not well understood. Polymerization forces, microtubule motor proteins, and myosin have all been implicated in microtubule bending. In porcine kidney epithelial cells stably expressing GFP- α -tubulin, we observed that microtubules were able to bend along their length even as their tips grew far away from the cell periphery. Crucially, this indicates that microtubule plus ends do not need to be held stationary for bends to form. We used photobleaching to create fiduciary markers along the lengths of microtubules under the nucleus and found that the majority of microtubule bends were formed by plus-end directed (anterograde) translation of a microtubule segment towards another, stationary microtubule segment, or pinning point. Simulations predicted that bends of the sizes observed in living cells cannot be produced by tangential forces without the presence of a pinning point. Pinning points were found to be transient in nature, sometimes releasing a microtubule and allowing for relaxation of an adjacent bend past where the microtubule was initially pinned. Inhibition of dynein caused a reduction in the proportion of bends caused by anterograde translation as well as in the overall incidence of microtubule bending, suggesting that dynein is providing the tangential force necessary to bend microtubules at pinning points. Myosin inhibition, on the other hand, had no effect on anterograde bending of microtubules. We propose a mechanism by which bending at pinning points can contribute to dynein-dependent deflection of growing microtubule tips. A growing plus end near a pinning point rotates around the pinning point as a bend forms. As the tip continues to grow, friction with the surrounding cytomatrix stabilizes its new direction, so that even if the pinning point dissipates and the bend relaxes, the microtubule cannot relax completely to its original direction. Simulations verified that pinning points near growing plus ends and dynein motor activity are sufficient to cause changes in microtubule direction.

P962**Board Number: B283****The yeast kinesin Kip2p promotes microtubule growth through polymerase and anti-catastrophe activities.**A. Hibbel¹, A. Bogdanova¹, D. Liakopoulos², J. Howard³;¹Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany, ²CRBM-CNRS, Montpellier, France, ³Molecular Biophysics and Biochemistry, Yale University, New Haven, CT

The lengths of individual microtubules need to be tightly controlled for cytoskeletal functions such as mitotic spindle positioning. Length control requires a precise balance between microtubule growth and shrinkage, but how this is achieved is poorly understood. In budding yeast, the orphan kinesin Kip2p is suggested to promote microtubule growth, as deletion of this kinesin results in shorter, less abundant cytoplasmic microtubules. However, how Kip2p promotes microtubule growth is not known.

We studied the effect of Kip2p on microtubule dynamics *in vitro*, using reconstitution assays combined with total internal reflection fluorescence (TIRF) and differential interference contrast (DIC) microscopy. In the presence of ATP, Kip2p increases the growth rate and inhibits the catastrophe rate of microtubules grown from either pig or yeast tubulin. These activities increase the mean length of dynamic microtubules, thereby promoting microtubule growth.

To investigate the molecular mechanism by which Kip2p controls microtubule dynamics, we determined biophysical properties of Kip2p on stabilized and dynamic microtubules in single-molecule experiments. In the presence of ATP, Kip2p is a highly processive motor that transports tubulin to the microtubule plus-end. In the presence of the non-hydrolyzable ATP analog AMP-PNP, Kip2p is stationary on the lattice and microtubule growth is no longer promoted. The flux and end-residence time of Kip2p on dynamic microtubules indicate that the mechanism by which Kip2p promotes microtubule growth is primarily by tubulin subunit addition while Kip2p is at the microtubule plus-end. The targeting of tubulin to the end by Kip2p plays a relatively small role in growth promotion.

Our results provide evidence that a kinesin motor can act directly as a microtubule polymerase and anti-catastrophe factor in the absence of accessory proteins.

P963**Board Number: B284****Human Kinesin-8 Kif18A is a Microtubule Depolymerase and Catastrophe Factor.**H. Petzold¹, J. Howard²;¹MPI-CBG, Dresden, Germany, ²Molecular Biophysics and Biochemistry, Yale University, New Haven, CT

Members of the kinesin-8 family of microtubule motors regulate the lengths of mitotic spindles in yeast, fly and mammalian cells. Kip3p, the budding yeast kinesin-8, has microtubule-depolymerizing and catastrophe-promoting activities *in vitro*, activities which accord with Kip3p's ability to regulate

microtubule length. By contrast, there are contradictory results in the literature on the activity of Kif18A, the mammalian kinesin-8: some labs have reported that it depolymerizes microtubules while others have reported that it inhibits catastrophe by pausing microtubule growth. These contradictory results raise the possibility that kinesin-8 function is not conserved, despite the high sequence similarity.

To elucidate how Kif18A affects microtubule dynamics, we studied its effect on stabilized and dynamic microtubules *in vitro*, using reconstitution assays combined with total internal reflection fluorescence (TIRF) microscopy. We found that, when added to dynamic microtubules, Kif18A increases the catastrophe rate in a concentration- and ATP-dependent manner. Furthermore, Kif18A depolymerizes GMPCPP- and taxol-stabilized microtubules.

Our results show that Kif18A is a microtubule depolymerase and catastrophe factor like its budding yeast ortholog Kip3p.

P964

Board Number: B285

EB1 and cytoplasmic dynein mediate protrusion dynamics for efficient three-dimensional cell migration.

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Microtubules have long been targeted to control tumor growth and, more recently, metastatic disease, for which a critical step is the local invasion of tumor cells into the 3D collagen-rich stromal matrix. Here we show that to migrate in collagen matrices human fibrosarcoma and breast cancer cells exploit the dynamic formation of highly branched protrusions, which are composed of a microtubule-filled core surrounded by actin filaments that is largely absent in the same cells flattened on 2D substrates. Microtubule plus-end tracking protein EB1 and microtubule-associated motor protein dynein critically modulate 3D cell migration, not by regulating vesicular trafficking, but by regulating both speed and persistence through regulation of protrusion branching itself regulated by differential assembly dynamics of microtubules in the protrusions. These proteins do not regulate conventional 2D migration. An important consequence of the prominent role of microtubules in 3D migration is that the treatment of fibrosarcomas by commonly used cancer drug paclitaxel, which stabilizes microtubules, is dramatically more effective in 3D than in 2D, uniformly and completely blocking 3D cell migration. This work reveals the central role that microtubule dynamics plays in cell migration in more pathologically-relevant 3D collagen matrices and suggests that cancer drugs targeting microtubule dynamics to mitigate migration should be further tested in 3D microenvironments.

P965**Board Number: B286****Understanding the regulation of +TIP complexes of microtubules.**K. Trogden¹, S. Rogers²;¹Biology, University of North Carolina, Chapel Hill, NC, ²Department of Biology, University of North Carolina, Chapel Hill, NC

Dynamic microtubules are necessary for many cellular functions, including cell motility, morphogenesis, and mitosis. MT dynamics are regulated by a conserved set of proteins, termed +TIPs, which interact primarily with the MT plus end. +TIPs encompass a number of protein families; some promote MT dynamics while others stabilize MTs. In *Drosophila*, the scaffolding proteins EB1 and Sentin promote MT dynamics by recruiting the TOG domain protein Mini spindles (Msps). Msps acts an "anti-pause" factor in S2 cells. It is known that the *Xenopus* homolog, XMAP215, can both processively add tubulin to growing ends and drive MT depolymerization in vitro and are thought to have the same activities in cells. Orbit is another +TIP that functions to stabilize MTs by suppressing dynamic instability. The goal of my project is to understand how the cell regulates these antagonistic activities using *Drosophila* S2 cells as a model system. Our data indicate that the ability of +TIPs to interact are regulated in S2 cells. This regulation, in part, is through the kinase GSK3 β downstream of Rac. GSK3 β can affect the localization of Orbit, similar to what has been seen in mammalian cells in which the Orbit homolog CLASP has been shown to be directly phosphorylated by GSK3 β . We found that in *Drosophila*, this pathway is conserved and also regulates Msps localization. Downstream of this pathway, Orbit is necessary to allow Msps to switch from tip to lattice localization of the microtubule. We also show that Msps and Orbit can interact through the scaffolding protein Sentin. We hypothesize that this interaction is important for proper microtubule dynamics in cells.

P966**Board Number: B287****Spatiotemporal control of microtubule dynamics by patterned illumination.**J. van Haren¹, H. Wang², K.M. Hahn², T. Wittmann¹;¹Department of Cell and Tissue Biology, University of California, San Francisco, San Francisco, CA,²Department of Pharmacology, University of North Carolina, Chapel Hill, NC

Reorganization of membranes, organelles and other intracellular components during complex cell dynamics such as cell division or directed migration relies on a tight spatial and temporal control of microtubule (MT) dynamics and interactions. Here we present three different light-activated microtubule modulators that we have designed to interrogate the functional relevance of local regulation of microtubule dynamics and interactions. +TIPs are a diverse class of proteins that associate specifically with the ends of growing MTs, and regulate MT dynamics, or mediate MT interactions with other intracellular structures such as kinetochores, organelles and the cell cortex. +TIPs therefore play an important role in spatially organizing the cell. End-binding proteins (EBs) are key elements of the

growing MT end, as these +TIPs recruit the majority of other +TIPs to MT ends. Several other EB-dependent +TIPs have been found to localize to other structures besides the MT end, such as for example CLASPs that localize to the cell cortex independently of MTs and capture MTs at the cell cortex. The interaction of CLASPs with MT ends at the cell cortex is spatially controlled by a gradient of GSK3- β activity. Polarized distribution/activation of +TIPs is likely a general mechanism for the establishment of a polarized MT network, and hence polarized cellular responses. Utilizing blue light controllable dimerization modules, LOV2/ZDK1 and Cry2/CIB1, we constructed light-controllable EBs, which we can use to spatiotemporally control intracellular +TIP or microtubule interactions. In addition, MT polymerization dynamics are spatiotemporally regulated in cells, and we utilized light-controlled inhibition of the small MT destabilizer Op18/stathmin to directly influence MT polymerization. These light-controlled MT modulators have advantages over knockdown approaches, since our systems can be used to locally control microtubule dynamics, and are fully reversible, and will allow to directly ask longstanding questions about intracellular microtubule function. We are currently testing how local control of MT dynamics by patterned illumination affects cellular behaviors.

P967

Board Number: B288

Role of TACC3 in Axon Elongation and Guidance.

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Axon outgrowth and guidance are two essential phenomena in establishing proper neural connection for a functioning nervous system. Navigating to the proper target requires the communication and coordination of the environmental guidance cues and cytoskeletal polymers at the growing tip of the axon, the growth cone. Microtubules are dynamic cytoskeletal polymers in the growth cone. The dynamic behavior of the microtubule is regulated by its plus end and a conserved family of proteins called 'plus-end tracking proteins' (+TIPs) located on these ends.

Our focus is to understand how +TIPs regulate dynamic behavior of microtubules during axon outgrowth and guidance. We are investigating the role of the +TIP TACC3 in axon outgrowth and guidance. Here we show that TACC3 is expressed in vertebrate neuron. Overexpression of it increases axon length while knocking it down reduces both axon length and number in *Xenopus laevis* growth cones. In addition to its effect on axon outgrowth, we also investigated its role in axon guidance. We performed in vitro stripe assays in which the neural tube and eye explants from *Xenopus laevis* embryos were cultured in a dish in the presence of stripe of the repulsive axon guidance cue, ephrin. The responsiveness of the growth cones of these explants were evaluated quantitatively by comparing the localization behavior of the growth cones as it navigates through the ephrin stripes. Our findings reveal that TACC3 functions as an axon outgrowth promoting factor in vivo in embryonic spinal cord neurons and it is required for proper axon navigation during optic nerve projection to the brain.

P968**Board Number: B289****TACC3 is a microtubule plus-end tracking protein that promotes axon elongation and also regulates microtubule plus-end dynamics in multiple embryonic cell types.**B.U. Nwagbara¹, B. Erdogan¹, E. Bearce¹, P. Ebbert¹, M. Evans¹, E. Rutherford¹, L.A. Lowery¹;¹Biology, Boston College, Chestnut Hill, MA

Proper neural connections, essential to nervous system function, depend upon precise navigation by the neuronal growth cone. A fundamental problem in growth cone cell biology is how guidance pathways are integrated to coordinate cytoskeletal dynamics, thus driving accurate steering. To address this question, we focus on the plus-ends of microtubules, which explore the growth cone periphery and play a key role in growth cone steering. Microtubule plus-end dynamics are regulated by a conserved family of proteins called 'plus-end-tracking proteins' (+TIPs). Yet, it is still unclear how +TIPs interact with each other and with plus-ends to control microtubule behavior, especially in the developing nervous system.

The centrosome-associated protein TACC3, a member of the transforming acidic coiled coil (TACC) domain family, has been previously implicated in regulating several aspects of microtubule dynamics. However, TACC3 has not been shown to function as a +TIP in vertebrates. Here, we show that TACC3 promotes axon outgrowth and also regulates microtubule dynamics by increasing microtubule plus-end velocities in vivo. We also demonstrate that TACC3 acts as a +TIP in multiple embryonic cell types, and that this requires the conserved C-terminal TACC domain. Using high-resolution live-imaging data of tagged +TIPs, we reveal that TACC3 localizes to the extreme microtubule plus-end, where it lies distal to the microtubule polymerization marker, EB1, and directly overlaps with the microtubule polymerase, XMAP215. TACC3 also plays a role in regulating XMAP215 stability and localizing XMAP215 to microtubule plus-ends. Together, our results implicate TACC3 as a +TIP that functions with XMAP215 to regulate microtubule plus-end dynamics.

P969**Board Number: B290****Mcp1p tracks microtubule plus ends to destabilize microtubules at cell tips.**F. Zheng¹, T. Li¹, M. Cheung¹, V. Syrovatkina², C. Fu³;¹Department of Biochemistry, Univ Hong Kong, Pokfulam, Hong Kong, ²Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA, ³Department of Biochemistry, Univ Hong Kong, Hong Kong, Hong Kong

Microtubule plus ends are dynamically regulated by a wide variety of proteins for performing diverse cellular functions. Here, we show that the fission yeast *Schizosaccharomyces pombe* uncharacterized protein mcp1p is a microtubule plus-end tracking protein which depends on the kinesin-8 klp6p for

transporting along microtubules towards microtubule plus ends. In the absence of mcp1p, microtubule catastrophe and rescue frequencies decrease, leading to an increased dwell time of microtubule plus ends at cell tips. Thus, these findings suggest that mcp1p may synergize with klp6p at microtubule plus-ends to destabilize microtubules.

P970

Board Number: B291

The novel microtubule-regulating protein, MTCL1, crosslinks and stabilizes noncentrosomal microtubules on the Golgi membrane.

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Recent studies have revealed the presence of a microtubule (MT) subpopulation called Golgi-derived MTs that support the Golgi ribbon formation required for maintaining polarized cell migration. CLASPs and AKAP450/CG-NAP are involved in their formation, but the underlying molecular mechanisms remain unclear. We have recently identified a novel MT-regulating protein, MTCL1, which crosslinks MTs through its N-terminal MT-binding region and subsequent coiled-coil motifs, and revealed its indispensable roles in accumulating the noncentrosomal MTs along the lateral membrane of polarized epithelial cells. In the current study, we demonstrate that MTCL1 colocalizes with the Golgi membrane through interactions with CLASPs and AKAP450/CG-NAP, and plays indispensable roles in the development of the Golgi-derived MTs. Correspondingly, MTCL1-depleted cells fail to develop the stable perinuclear MTs and Golgi ribbon. Interestingly, not only the N-terminal MT-binding region, which promotes MT accumulation around the Golgi membrane, but also the C-terminal MT-binding region showing a novel MT-stabilizing activity, are required for rescuing these defects in MTCL1-depleted cells. Taken together, the present results suggest that MTCL1 cooperates with CLASPs and AKAP450/CG-NAP for the formation of the Golgi-derived MTs, and mediates their development into a stable Golgi-associated MT network along which the Golgi stacks extend to form the Golgi ribbon. These results reveal a novel role of MTCL1 in organizing another kind of noncentrosomal MTs and clarify how the Golgi-associated MTs are specifically stabilized.

P971**Board Number: B292****A Role for Microtubules in Lens Fiber Cell Elongation and Lens Morphogenesis.**C.M. Logan¹, L. Zhang¹, S. Menko²;¹Anatomy, Thomas Jefferson University, Philadelphia, PA, ²Pathology, Anatomy and Cell Biology, Thomas Jefferson University, Philadelphia, PA

Tissue development and regeneration involve high-ordered morphogenetic processes that are governed by elements of the cytoskeleton in conjunction with cell adhesion molecules. Such processes are particularly important in the lens whose structure dictates its function. Microtubules have many roles in the cell, among them as determinants of directional migration and as the highways for vesicle transport. Here we investigated the possible role of microtubules and their interactions with N-cadherin in providing directionality to fiber cell elongation. Through co-immunoprecipitation analysis using chick embryo lenses microdissected into four distinct zones of differentiation we demonstrated that N-cadherin interacts with tubulin and acetylated-tubulin primarily in the cortical fiber zone, where lens fiber cells elongate. Disassembly of microtubules with the microtubule depolymerizing drug nocodazole affected lens fiber cell elongation and directionality. High doses of nocodazole also affected interactions between fiber cells and epithelial cells along the epithelial-fiber interface (EFI). These effects were accompanied by a dramatic loss of association between N-cadherin and acetylated tubulin in the cortical fiber zone, as well as changes in levels of F-actin and phospho-myosin. These results provide the first demonstration of a role for microtubules in lens fiber cell elongation, lens morphogenesis and the maintenance of lens tissue integrity.

P972**Board Number: B293****Persistence of microtubule polymerization controls cell migration in 3D matrix.**B. Bouchet¹, I. Noordstra¹, A. Akhmanova¹;¹Cell Biology, Faculty of Science, Utrecht University, Utrecht, Netherlands

Cooperation between the actin and microtubule cytoskeleton is essential for cell migration, but the underlying mechanisms differ depending on the cell type and cell culture conditions. In 2D cultures, actin polymerization typically drives spreading of lamella while actomyosin-based contractility translocates the cell body towards the leading edge. In this context, microtubules are thought to have primarily a regulatory function linked to the control of signaling and focal adhesion dynamics. In more physiologically relevant 3D culture models, cells do not form flat lamella but instead can extend long pseudopods that penetrate the matrix and form adhesions. Also in these conditions, the actomyosin network is essential for cell protrusion and translocation, but the role of microtubules is still poorly understood. Here, we investigated whether microtubule dynamics is an important factor in 3D migration. We used inactivation of SLAIN2, a recently identified interphase-specific plus-end recruitment factor for the microtubule polymerase ch-TOG, to disrupt persistent microtubule polymerization. When

SLAIN2 is inactivated, interphase microtubules grow slowly and undergo frequent catastrophes, but the organization and maintenance of microtubule network is relatively normal and cell divide normally, because SLAIN2 plays no role in the formation of the mitotic apparatus. Surprisingly, we found that processive microtubule growth is dispensable for cell spreading and migration in 2D, but is completely essential for the mesenchymal cell movement in 3D matrix. When persistent microtubule polymerization is reduced, the formation of long pseudopods that initiate cell movement in 3D is dramatically inhibited. Short term treatments with very low doses of microtubule-targeting agents that induced similar perturbation of microtubule growth without destroying microtubule network caused rapid retraction of the existing pseudopods, indicating that also the maintenance of cell protrusions in 3D depends on persistence of microtubule growth. We further found that rapid microtubule polymerization antagonizes myosin II-based contractility during pseudopod extension in 3D matrix. Finally, we showed that persistent microtubule growth is a prerequisite for pseudopod-based invasion induced by complete epithelial-mesenchymal transition (EMT) in human breast epithelial cells, a process that is crucial for both normal development and cancer cell metastasis. Our results demonstrate that, independently from microtubule network maintenance, the microtubule polymerization processivity is a critical factor required for the elongation of pseudopods that initiate cell motility in 3D matrix.

P973

Board Number: B294

Paxillin interacts with and inhibits HDAC6 to regulate microtubule acetylation, Golgi integrity and polarized cell migration.

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Polarized cell migration is of fundamental importance for organism development and maintenance, including tissue patterning, wound healing and immune surveillance. However, the dissemination of cancer cells from the primary tumor to generate distant metastases also requires navigation of cells through the tissue stroma to the lymphovascular system. A hallmark of directional migration is the repositioning of a cohesive Golgi apparatus ahead of the nucleus to facilitate trafficking of pro-migratory factors to the appropriate cellular locales. Furthermore, spatiotemporal regulation of dynamic cell-extracellular matrix interactions is also essential for cells to generate the traction forces necessary for cell movement. Importantly, these aforementioned key events in directional migration can all be regulated by the microtubule cytoskeleton. Paxillin localizes to sites of cell-extracellular matrix interaction, termed adhesion contacts, where it serves to integrate a diverse array of signaling and structural proteins. Historically, paxillin has been shown to function at adhesion contacts to predominantly serve as a hub for Rho GTPase family protein signaling to coordinate the actin cytoskeleton. Herein we report a new role for paxillin in regulating the microtubule network and cell polarization machinery through its interaction with and inhibition of HDAC6 in both normal and cancer cells. Using a combination of FRET and proximity ligation assays we determined that while a small percentage of the paxillin-HDAC6 complexes were found in and around adhesion contacts, the majority

was distant from these sites of robust paxillin enrichment. Furthermore, through inactivation of HDAC6, paxillin, is able to regulate polarized cell migration/invasion in both 2D and 3D microenvironments by controlling the microtubule acetylation-dependent cohesion and repositioning of the Golgi apparatus. We also determined that the proline-rich region of paxillin is necessary for the interaction with and inhibition of HDAC6. This study has identified a new and ubiquitous role for paxillin as a key regulator of the microtubule network and cell polarization machinery.

P974

Board Number: B295

Effects of Rev Protein on Microtubule Arrays in Living Cells.

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The HIV protein Rev regulates the expression of essential viral proteins during the course of infection by a mechanism that is well understood. It promotes nuclear export of viral transcripts, normally retained in the nucleus owing to the presence of introns, by interacting with host cell transport factors. However, over-expression of Rev in cells leads to defects in cell cycle progression, specifically slowing growth and impairing progression through mitosis (Miyazaki et al., 1995, Exp. Cell Res. 219:93-101, data shown here). While it is possible that Rev may be altering the proteins in transport pathways, cell cycle defects may be attributed to Rev's interactions with other proteins.

In vitro experiments show that highly purified Rev has a high and specific affinity for α and β tubulin present either as free heterodimers or polymerized into microtubules (MTs) (Watts et al., 2000, J. Cell Biol. 150:349-360). Moreover, Rev rapidly depolymerizes MTs *in vitro* producing intermediates that closely resemble the products of depolymerization reactions triggered by a variety of experimental conditions. Owing to structural similarities, Rev hypothetically depolymerizes MTs by a mechanism used by Kinesin-13 (Kin-13) proteins that are potent MT depolymerizing enzymes.

To determine whether Rev is interacting with MTs in a Kin-13-like manner, point mutations were introduced into Rev substituting alanine for amino acids shared with Kin-13. Mutant proteins were tagged with YFP, over-expressed in HeLa cells and cell cycle progression was monitored. In contrast to expression of Rev, which lengthened doubling times and all stages of the cell cycle, each point mutant partially corrected the defect. These results are consistent with Rev acting in a manner similar to Kin-13.

To determine whether Rev over-expression affects MT dynamics in cells, MT arrays were experimentally depolymerized and allowed to recover. If Rev inhibits MT nucleation or promotes depolymerization, then MT arrays in cells expressing Rev should require more time to recover. Results show that MT arrays recover from depolymerization equally well in presence and absence of Rev. Because wild-type Rev accumulates largely in the nucleus and nucleoli, we attempted to increase the cytoplasmic levels of Rev by introducing M4 and M6 mutations that impair Rev multimerization and nuclear import, respectively. However, neither M4 nor M6 expression affected MT recovery after depolymerization.

These results suggest that the cell cycle defects observed in Rev-expressing cells are not mediated by Rev's ability to alter the polymerization state of MT.

P975

Board Number: B300

NuSAP stabilizes kinetochore microtubules by attenuating MCAK depolymerization activity.

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NuSAP (Nuclear and Spindle Associated Protein), a novel Microtubule Associated Protein (MAP), functions as a microtubule stabilizer. Depletion of NuSAP leads to severe mitotic defects. However, it remains unclear how NuSAP stabilizes microtubules in cells during mitosis. In this paper, we dissect the function of NuSAP in stabilizing kinetochore microtubules during metaphase and present the first evidence of the direct interaction of NuSAP and a MT depolymerizer, MCAK. NuSAP tightly regulates the localization, dynamics and depolymerization activity of MCAK. Furthermore, we demonstrate that Aurora B kinase regulates the functional complex of NuSAP and MCAK at kinetochore region. Aurora B significantly enhances the interaction of NuSAP with MCAK and further modulates the effects of NuSAP on the localization and depolymerization activity of MCAK. Thus, we provide new insights into the complex regulation of kinetochore microtubules dynamics during metaphase to ensure proper tension at kinetochore and establish the attachment of kinetochore microtubules to promote precise cell division.

P976

Board Number: B301

Kinetochore microtubule dynamics are controlled by microtubule length and centromere tension forces in the *S. cerevisiae* mitotic spindle.

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During metaphase, dynamic kinetochore microtubules grow and shrink to align sister chromosomes on opposite halves of a mitotic spindle. Proper chromosome alignment is necessary to ensure their faithful segregation to daughter cells during anaphase, and requires fine-tuning of the kinetochore microtubule transition frequencies between growing and shortening states (rescue and catastrophe). In budding yeast, both mechanical tension as well as microtubule length-dependent regulatory proteins likely contribute to this alignment process. However, the relative importance of these effects remains unclear. Thus, we quantified the dynamics of single kinetochore microtubules with high temporal and spatial resolution to determine the relative contributions of microtubule length and tension force to metaphase kinetochore positioning. Quantification of dynamics in wild-type cells demonstrated that microtubule

switching frequencies were affected by both microtubule length and tension, although microtubule length had a more substantial effect on transition frequencies than the effect of tension. Regardless, increased tension values were correlated with both increased rescue frequency and reduced catastrophe frequency, and longer microtubule lengths were correlated with both reduced rescue frequency and increased catastrophe frequency. We conclude that both tension and kinetochore microtubule length contribute to metaphase kinetochore positioning in budding yeast.

P977

Board Number: B302

The CHE-12 protein family uses a TOG domain array to regulate microtubules in the primary cilium.

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Primary cilia are microtubule-based projections from the cell surface, critical for normal development, sensation, and homeostasis. A solitary primary cilium is present in most mammalian cell types, where it acts as a cellular antenna to sense the extracellular environment. Defects in cilia architecture and function yield a host of diseases collectively termed ciliopathies. Cilia consist of basal, middle and distal zones, characterized by triplet, doublet and singlet microtubules, respectively. The catalog of factors that regulate ciliary microtubules and their respective mechanisms are poorly understood. The XMAP215 and CLASP families are key TOG domain array-based regulators of cytoplasmic microtubules, but whether TOG array proteins regulate cilia structure and function remains to be determined. We have identified a conserved protein family unique to ciliated/flagellated organisms that use a tetrameric TOG domain array to regulate ciliary microtubules, defined by the *C. elegans* member CHE-12. *che-12* worms lack singlet microtubules in cilia distal segments and have defective doublet architecture, resulting in aberrant chemotaxis. We present the crystal structure of mammalian CHE-12 (mCHE-12) TOG2 revealing a canonical TOG fold with conserved tubulin-binding determinants. We have shown that mCHE-12 localizes to the distal tip of primary cilia in mammalian cell culture and can also bind along cytoplasmic microtubules when over-expressed. Using a light scattering assay, we demonstrate that mCHE-12 TOG domains promote microtubule polymerization in vitro. We used the recently developed Cas9-triggered homologous recombination method to introduce mutations in the endogenous *che-12* genetic locus in *C. elegans*, and demonstrated that CHE-12's TOG domain-dependent tubulin-binding activity is essential for proper amphid sensory cilia development. Taken together, our findings suggest that CHE-12 uses its TOG domain array to bind tubulin and regulate microtubule organization in primary cilia. Thus, the CHE-12 family expands the TOG domain array-based MT regulatory paradigm with key implications for cilia structure and function.

P978**Board Number: B303****CLASP1 Differentially Regulates Microtubule Growth Dynamics in Response to ECM Composition and Density.**N. Caesar¹, K.A. Myers¹;¹Biological Sciences, University of the Sciences, Philadelphia, PA

Angiogenesis is the process by which endothelial cells (ECs) are signaled to polarize, extend branches, and migrate with directional specificity to establish new vascular networks from existing vasculature. To establish a polarized morphology, ECs extend branched protrusions, a process that requires the differential regulation of microtubule (MT) dynamics. Regulation of MT dynamics is accomplished by Microtubule Associated Proteins (MAPs), which function by promoting MT growth, MT stability, or MT disassembly. CLASP1 is a MAP that associates with MTs with regional specificity to promote MT stability, and in doing so contributes to cell polarization. In addition to MAPs, interactions with the extracellular matrix (ECM) are known to differentially influence MT dynamics. For example, variations in ECM dimensionality and compliance cause marked differences in MT growth dynamics, resulting in altered EC branching morphologies. Nevertheless, it is not known if, or how, CLASP1 contributes to ECM-engagement-mediated regulation of MT growth. We tested the hypothesis that CLASP1 promotes the regional regulation of MT growth dynamics in response to ECM engagement in order to drive EC branching. HUVECs were cultured on either high-density (90ug/mL) or low-density (45ug/mL) 2D collagen-I ECMs, and were compared to HUVECs cultured on either high-density (100ug/mL) or low-density (10ug/mL) 2D fibronectin ECMs. Our results show that HUVEC engagement of fibronectin ECMs resulted in significantly faster and longer-lived MT growth than engagement of collagen, independent of ECM density. On fibronectin ECMs, CLASP1 expression resulted in significantly slower and shorter-lived MT growth, independent of ECM density. In HUVECs cultured on low-density collagen ECMs, CLASP 1 expression promoted fast MT growth with no effect on MT growth lifetimes. However, on high-density collagen ECMs, CLASP1 expression promoted significantly slower and longer-lived MT growth. Regional analysis comparing the branched periphery versus the cell center revealed that CLASP1 expression promoted slower MT growth speeds in the cell periphery but had no effect on MT growth lifetimes, independent of ECM composition or ECM density. Analysis of HUVEC branching revealed that only HUVEC engagement of high-density fibronectin ECMs significantly increased branch number, and that CLASP1 expression on high-density fibronectin ECMs further increased branch number. Together, these results suggest that CLASP1 differentially regulates MT dynamic instability in response to both ECM composition and ECM density, and suggest that CLASP1 regional regulation of MT growth dynamics contributes to HUVEC branching by promoting slow and short-lived MT growth in response to HUVEC engagement of high-density fibronectin ECMs.

P979**Board Number: B304****Dissecting the role of microtubule poleward flux in correction of kinetochore mis-attachment.**E. Roscioli¹, B. He¹, H. Bomberger¹, M. Shen¹, G. Civelekoglu-Scholey², D. Cimini¹;¹Biological Sciences, Virginia Bioinformatics Institute, Virginia Tech, Blacksburg, VA, ²Department of Molecular and Cellular Biology, University of California, Davis, CA

Microtubule (MT) poleward flux refers to a specific aspect of MT dynamics by which individual tubulin subunits within mitotic spindle MTs translocate poleward. This behavior is thought to arise from MT depolymerization at the minus end (spindle pole), MT polymerization at the plus end, and concomitant motor-dependent poleward sliding of the MT lattice. In this process, kinesin 13s (KLP10A in *Drosophila*, Kif2a in vertebrates) at the spindle poles act as the major MT minus end depolymerases. The biological role of MT poleward flux has been long debated, given that it is only observed in certain model systems. Previous studies indicated that suppression of poleward flux results in an increased rate of chromosome mis-segregation in the form of anaphase lagging chromosomes (Ganem and Compton, *Curr. Biol.*, 2005) and increased desynchronization of chromosome poleward movement (by distribution of poleward forces across the spindle; Matos et al., *J. Cell Biol.*, 2009). However, in the study by Ganem and Compton, suppression of poleward flux could only be performed in cells co-depleted of the plus end depolymerase MCAK and, in Matos et al., KLP10A and CLASP were knocked down simultaneously and their quantitative model did not account for MT dynamic instability or kinetochore (KT) attachment state. We have recently developed a comprehensive mathematical model of chromosome dynamics that includes MT plus and minus end dynamics and poleward flux, as well as an experimental approach (microinjection of Kif2a antibodies) to suppress MT poleward flux independent of other perturbations. Here, we combined these approaches to investigate the role of MT poleward flux in correction of KT mis-attachment. Computer simulations of metaphase in the absence of poleward flux indicated that KT-MT attachments become more stable, resulting in higher numbers of kinetochore MTs (kMTs) with increased kMT half-life. Given that kMT turnover is required for the correction of KT mis-attachments, these results suggest that inhibition of poleward flux alone is enough to impair this mechanism of correction. To test this prediction, we examined chromosome segregation by time-lapse microscopy in cells microinjected with Kif2a antibodies and found that more than 40% (15/35) of the cells displayed anaphase lagging chromosomes as opposed to 13% (4/30) in buffer-injected cells. These results strongly support the idea that MT poleward flux plays a key role in correction of KT mis-attachment. We are currently investigating additional effects suppression of MT poleward flux may have on overall organization of the metaphase spindle.

P980**Board Number: B305****Xenopus Dppa2 is an embryo-specific inhibitor of microtubule growth required for nuclear assembly.**J. Xue¹, M. Takata¹, H. Funabiki¹;¹Laboratory of Chromosome and Cell Biology, Rockefeller University, New York, NY

Dppa2 is a member of a gene family expressed exclusively in embryonic and pluripotent tissues, originally cloned through coexpression with Oct4. Dppa2 orthologs are found throughout vertebrates, and we identified *Xenopus* Dppa2 in a proteomic screen for novel chromatin-binding proteins. Interestingly, despite its exclusive localization to chromatin, Dppa2 contains a microtubule-regulatory activity in its C-terminal domain spanning 97 amino acids. Here we present our studies analyzing the effects of recombinant Dppa2 on the dynamics of in vitro microtubules. In a dose-dependent manner, Dppa2 inhibits growth from pre-existing microtubule seeds and appears to suppress rescue frequency. In vivo, Dppa2 disassembles the remnants of metaphase microtubules around chromatin at the transition from metaphase to interphase and is required for the assembly of normal, replication-competent nuclei. In the absence of Dppa2, nuclei are smaller and have distorted morphology. This phenotype is mimicked by taxol treatment and rescued by nocodazole, confirming the importance of regulated microtubule dynamics during nuclear assembly. Dppa2 expression is limited to the early *Xenopus* embryo, which is notable both for its large size (1 mm diameter) and its rapid cell divisions (once every 30 minutes). We propose that rapid cell cycles in large cells pose unique challenges to the mitotic apparatus, necessitating specific solutions that maintain tight spatial and temporal control of cytoskeletal dynamics.

P981**Board Number: B306****SUMO-2/3 modification regulates the stability of microtubules.**S. Hong¹, W. Law¹, K. Middleton¹;¹Cytoskeleton, Inc., Denver, CO

Microtubule undergoes post-translational modifications (PTMs), which diversify its functional regulations on intracellular transport, cell shape, motility and differentiation. Various PTMs on microtubules are well documented in literature, but SUMOylation of microtubule has not been clearly demonstrated so far. In this study, we have detected microtubule structure, SUMOylated by endogenous SUMO-2/3, in HeLa cells. For the first time, we visualized cytoplasmic network, targeted by SUMO-2/3, with immuno-fluorescent staining. This network is densely populated near the perinuclear region and emanates to the periphery of cells, which is well co-localized with microtubules. Clearly, SUMO-2/3 modifies either directly microtubule (or tubulin) or microtubule-associated proteins (MAPs). Interestingly, this SUMOylated microtubule structure shows strong resistance against Nocodazole treatment (10uM for 1hr), which may indicate that SUMOylation by SUMO-2/3 is involved in formation

and/or maintenance of stable microtubules rather than dynamic ones. Up to now, it is not clear if SUMO-2/3 preferentially targets stable microtubules or if SUMO-2/3 modified tubulins form stable microtubules. However, our observation that paclitaxel treatment (Taxol, 1 μ M for 4hrs) does not increase the level of SUMOylated microtubule may indicate that the latter is more possible scenario than the former. Next, we treated HeLa cells with Trichostatin A (TSA, 5 μ M for 4hrs), which inhibits Histone Deacetylase6 (HDAC6). While acetylated microtubules are greatly increased and form solid structures, most of SUMOylated microtubules are fragmented and long cable-like networks disappeared. Taken together, our data shows that endogenous SUMO-2/3 modifies microtubule structure and this SUMOylation regulates the stability of microtubules. Further studies on the cross-talk of SUMOylation with other PTMs will provide insights into the regulatory mechanism of dynamics and stability of microtubule.

P982

Board Number: B307

TLL6 Promotes Slow MT Growth at the Leading Edge of Polarized Endothelial Cells.

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Angiogenesis is the formation of new vasculature from existing vasculature. During angiogenesis, endothelial cells migrate to specific locations to form new blood vessels. In order to migrate with directional specificity, endothelial cells (ECs) generate a polarized morphology by establishing a leading and trailing edge. EC polarization requires the coordinated re-organization of the microtubule (MT) array, a process that is accomplished through the careful regulation of MT dynamics by various families of MT associated proteins (MAPs). Mitotic Centromere Associated Kinesin (MCAK) is a MAP that binds to MT plus ends and induces MT disassembly. One method used by cells to control MCAK-mediated MT disassembly is through recruitment of MCAK to specific, post-translationally modified regions of the MT lattice. MCAK binding to MTs is promoted by tubulin tyrosination, while polyglutamylation of MTs inhibits MCAK association. Tubulin Tyrosine Ligase-Like 6 (TLL6) is an enzyme that functions to post-translationally glutamylate MTs; however, it is not known what effects TLL6-mediated glutamylation has on MT growth dynamics, or how TLL6-mediated glutamylation regulates MCAK-mediated MT disassembly. To investigate these questions, we performed high-resolution live-cell imaging of polarized, wound-edge Human Umbilical Vein Endothelial Cells (HUVECs) either expressing mApple-EB3 alone (Control) or co-expressing mApple-EB3 with TLL6 or MCAK. We analyzed MT growth speeds and growth lifetimes of whole cell, leading edge and trailing edge regions using an automated MatLab based MT tracking program. Our results show that TLL6 promoted slow MT growth speeds compared to Control cells or compared to cells expressing MCAK. Regional analysis of MT dynamics in the leading and trailing edges of wound-edge HUVECs revealed that MCAK expressing cells had significantly faster MT growth speeds specifically within the leading edge when compared to Control HUVECs. Additionally,

in TLL6 expressing HUVECs, leading edge MT growth speeds were significantly slower than trailing edge MT growth speeds, suggesting that TLL6 mediates differential regulation of MT growth dynamics at the leading and trailing edge of the cell. MCAK expression reversed this phenotype, by promoting fast MT growth within the leading edge of wound-edge HUVECs. Thus, MT glutamylation by TLL6 promotes slow and short-lived MT growth globally throughout the cell, while at the leading edge of the cell, TLL6 promotes the slowest MT growth speeds, and this effect can be reversed by MCAK overexpression. Together, these data suggest that TLL6 glutamylation may function to inhibit MCAK with regional specificity at the leading edge of wound-edge HUVECs.

P983

Board Number: B308

Compliance Myosin-II-dependent ECM Mechanosensing Regulates Microtubule Dynamics Through a MCAK-Mediated Signaling Pathway.

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Directed cell migration relies on soluble guidance cues, as well as, the physical/mechanical attributes of the extracellular matrix (ECM). Transmission of ECM physical properties, compliance (softness) and dimensionality, to intracellular signaling events occurs largely through reorganization of the actin-myosin and microtubule (MT) cytoskeletons. Changes in MT dynamics are mediated in part by MT-associated proteins such as Mitotic Centromere Associated Kinesin (MCAK), a MT depolymerizer, and changes in myosin-II-dependent contractility via ECM mechanosensing. Here, we examined the effect of MCAK expression levels on MT growth speed and growth lifetime, and branching morphology in Human Umbilical Vein Endothelial Cells (HUVECs) cultured on stiff (55 kPa) and soft (0.7 kPa) collagen-coupled polyacrylamide ECMs. MT assembly dynamics were analyzed using high-resolution live-cell microscopy and MT plus end-end tracking software. Our data demonstrate that on stiff ECMs, MCAK expression results in slow, shorter-lived MT growth while on compliant ECMs, MCAK expression resulted in fast MT growth but had no effect on growth lifetime. On stiff ECMs, inhibition of myosin-II contractility with 20 μ M blebbistatin resulted in fast, shorter-lived MT growth, independent of MCAK expression. However, on compliant ECMs, myosin-II inhibition resulted in slow, shorter-lived MT growth only in MCAK-expressing HUVECs. These results suggest that the effects of MCAK on MT growth speed and growth lifetime are sensitive to myosin-II contractility specifically in response to ECM compliance mechanosensing. Regional analysis of MT dynamics revealed that growth speeds were similar in branched regions as compared to the whole cell, while growth lifetimes were significantly longer-lived within HUVEC branches. This effect was independent of ECM compliance or MCAK expression. Analysis of MT growth dynamics within branched regions revealed that myosin-II inhibition promoted fast MT growth only on stiff ECMs, but did not significantly affect MT growth lifetimes. Under conditions of myosin-II inhibition, MCAK expression significantly reduced MT growth speeds independent of stiffness, but reduced MT growth lifetimes only on stiff ECMs. Analysis of branching morphology revealed that

MCAK expression increased branch number on stiff substrates but reduced branch number on compliant ECMs, while MCAK expression in myosin-II inhibited cells resulted in reduced branch number on both stiff and compliant ECMs. These data suggest that, within HUVEC branches, myosin-II-dependent regulation of MT growth dynamics functions to promote slow MT growth through a MCAK-dependent pathway. However, when myosin-II is inhibited on compliant substrates, MT growth lifetimes become insensitive to MCAK-mediated regulation.

P984

Board Number: B309

Hereditary Neuralgic Amyotrophy Mutations Alter the Microtubule Bundling Properties of Septin 9.

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Hereditary neuralgic amyotrophy (HNA) is a rare, autosomal dominant neuropathy that is characterized by sudden onset of severe pain in the shoulder girdle, together with arm and shoulder muscle degeneration (atrophy). To date, the only gene associated with HNA is septin9 (SEPT9). Missense mutations of SEPT9 (R88W and S93F) and partial duplication of SEPT9 sequence have been reported in HNA patients. Septins are oligomeric, GTP-binding proteins and SEPT9 was found to associate and bundle microtubules (MTs) and regulate asymmetric neurite growth. By incubating the SEPT9i3 or its mutants with pre-polymerized microtubules and sedimenting the complexes at 39,000xg (high speed pelleting) or 8,000xg (low speed pelleting), we tested the ability of the proteins to bind and bundle microtubules, respectively. The high speed pelleting assay shows that neither the missense mutations nor the partial duplication (aa19-233) of SEPT9i3 alter microtubule binding to SEPT9i3. The low speed pelleting assay suggests that R88W and S93F missense mutations significantly decrease microtubule bundling, relative to wild type levels. The SEPT9 and HNA associated mutations/microtubule relationship was also investigated with a visual assay. Rhodamine-MT was incubated with wild type SEPT9i3, SEPT9i3R88W, SEPT9i3S93F or SEPT9_i3(aa19-233). Wild type SEPT9i3 has been previously shown to induce bright and long MT bundles. SEPT9R88W/S93F show a decrease in length of MT bundles, and, alternatively, SEPT9i3(aa19-233) shows an increase in length of MT bundles relative to wild type SEPT9i3. Collectively, our data suggest that the HNA-associated mutations alter the MT bundling properties of SEPT9. On-going studies aim at determining the effects of HNA-linked SEPT9 mutants on neuronal morphogenesis and function.

P985**Board Number: B310****Tenovin-treated cells show defects in mitosis and cytokinesis and tubulin cytoskeleton abnormalities in interphase.**M. . Carmena¹, C. Breunig², O. Molina¹, I. van Leeuwen³, M. Ladds³, S. Lain³, W.C. Earnshaw¹;¹Wellcome Trust Centre for Cell Biology, The University of Edinburgh, Edinburgh, United Kingdom,²University of Bayreuth, Bayreuth, Germany, ³Karolinska Institutet, Stockholm, Sweden

Sirtuins (SirT) are NAD(+)-dependent acetyl-lysine deacetylases known to be involved in the control of cellular metabolism and maintenance of cellular homeostasis. Despite being classified as Class III histone acetyltransferases (HDACs) these enzymes act upon both histone and non-histone substrates. SirT1 was reported to regulate the transcriptional activity of p53, suggesting that Sirtuin inhibition could result in improved tumour suppression and leading to exploration of the therapeutic potential of Sirtuin inhibitors.

Tenovin 1, which was identified in a screen for small molecule activators of p53 is the founder member of the Tenovin family of Sirtuin inhibitors. Tenovin 6 (T6), a more potent, water-soluble analogue inhibits the activity of SirT1 and SirT2 *in vitro* (Lain et al, 1998). T6 reduces tumour growth *in vivo* (melanoma and neuroblastoma xenografts) and contributes to eliminate leukemic stem cells (Li et al 2012; Li et al., 2014). The Tenovin D3 derivative (TD3) shows higher specificity for SirT2 *in vitro* (McCarthy et al 2013).

We have determined the phenotypic consequences of inhibiting SirT1/SirT2 in human cultured cells (RPE-1 and HeLa) using T6 and TD3. Treatment of p53 defective cells with either compound does not lead to cell cycle arrest in interphase. Our studies revealed the presence of defects in both mitosis and cytokinesis in T6 and TD3-treated cells. The most common defect observed in treated cells was the presence of chromatin bridges in late mitosis and cytokinesis. We also detected misaligned chromosomes in prometaphase and lagging chromatids in anaphase at lower frequencies.

Although the actin cytoskeleton appeared normal in Tenovin-treated cells, we observed increased levels of acetylated tubulin (a SirT2 substrate) throughout mitosis, with levels particularly high at midbodies in cytokinesis. Interestingly, we found an increased frequency of binucleated cells in which nuclei remained connected by chromatin bridges. This phenotype was frequently accompanied by an abnormal accumulation of tubulin between the nuclei, consistent with an increased stability of the microtubules in this area. Drug-treated cells also showed elongated cytoplasmic extensions containing abnormal projections of acetylated tubulin.

Finally, we studied the effect of treatment with T6 and TD3 in the levels of Histone acetylation and we found that the levels of H3K9ac are significantly elevated in drug-treated cells compared to controls.

Our results shed new light on the phenotypic effects of Tenovin treatment in cell cycle progression and genomic stability in different cell lines.

References

Lain S. *et al* Cancer Cell. 13:454 (2008).

Li, L. *et al*. Cancer Cell. 21:266 (2012).

McCarthy AR. *et al*. Mol Cancer Ther. 12:352 (2013).

Li L . *et al*. Cell Stem Cell. 2:431(2014).

P986

Board Number: B311

A novel role for Drosophila KASH domain protein Klarsicht in microtubule stability during collective cell migration.

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During collective migration of the Drosophila embryonic salivary gland, cells rearrange to form a tube of a distinct shape and size. Here, we report a novel role for Drosophila KASH (Klarsicht-Anc-Syne-Homology) domain protein, Klarsicht (Klar), in the regulation of microtubule (MT) stability and integrin receptor localization during salivary gland migration. In wild-type salivary glands MTs become progressively stabilized as gland migration progressed. In klar mutant embryos, salivary gland cells failed to rearrange and migrate, and these defects were accompanied by decreased MT stability and altered integrin receptor localization. Overexpression of Spastin (Spas), a MT severing protein, in otherwise wild-type salivary glands disrupted cell rearrangement and integrin localization, like loss of klar. Promoting MT stability by reducing spas gene dosage in klar mutant embryos rescued the integrin localization, cell rearrangement and gland migration defects. Klar genetically interacts with the Rho1 small GTPase in salivary gland migration and is required for the subcellular localization of Rho1. We also show that Klar co-immunoprecipitates with tubulin from whole embryo lysates and binds tubulin directly in vitro. Our studies provide the first evidence for klar in regulation of the MT cytoskeleton and integrin localization during collective cell migration.

Assembly and Disassembly of Cilia/Flagella 2

P987

Board Number: B313

Protein phosphorylation of FLA8/KIF3B serves as a molecular switch to control IFT entry and turnaround.

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Intraflagellar transport (IFT), a bidirectional transport system between the ciliary base and tip, delivers ciliary precursors and turnover products for cilia assembly and maintenance as well as signaling molecules for signal transduction. The anterograde transport from the ciliary base to tip is powered by kinesin-II whereas the retrograde transport is driven by cytoplasmic dynein. The motors carries protein complexes called IFT particles comprising IFT-A and –B complexes that serves as cargo adaptors for ciliary components. IFT is regulated both at the ciliary base and ciliary tip. Kinesin-II is activated and carries IFT particles to enter the cilium at the ciliary base. At the ciliary tip, IFT particles are unloaded and kinesin-II is inactivated. However, the regulatory mechanism is unknown. We have found that CrCDPK1 phosphorylates kinesin-II motor subunit FLA8/KIF3B at the conserved residue S663 and regulates the activity of kinesin-II, and loading and unloading of IFT particles at the ciliary base and tip. We show that FLA8/KIF3B is a substrate of CrCDPK1, that IFT-B loading and kinesin-II/IFT ciliary entry require FLA8 dephosphorylation, and that FLA8 phosphorylation is needed for IFT-B unloading at the ciliary tip. Moreover, changes of the cellular level of phosphorylated FLA8 are associated with IFT entry rate during cilia assembly. These results suggest that phosphorylation of FLA8/KIF3B mediated by CrCDPK1 controls IFT entry and turnaround. .

P988

Board Number: B314

How to specialize a cilium: MT glutamylation and tubulin composition regulate axonemal ultrastructure.

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Cilia are morphology and functionally diverse microtubule (MT) based organelles that are vital for human development and health. To understand ciliary specialization, we compared two morphologically distinct cilia types in *C.elegans*: sinusoid shape cephalic male (CEM) and rod shaped amphid channel cilia. MT ultrastructure of CEM and amphid cilia was significantly different. The transition zone (TZ) consists of nine MT doublets, comprised of a 13-protofilament A-tubule and, 11-protofilament B-tubule,

anchored to the membrane by proteinaceous Y-links. TZ immediately give rise to the 'middle segment', in which nine MT doublets devoid Y-links. Middle segment give rise to the distal segment, which is comprised of MT singlets and projects to the tip of the cilium. These singlets are the A-tubules of the MT doublets. B-tubules in MT doublets abruptly stop at the end of the middle segment.

In contrast to the amphid cilium, the CEM cilium has a longer TZ and a shorter middle segment. Nine MT-doublets in the middle segment give rise to 18 MT-singlets, which arise via splitting and extension of both A- and B-tubules doublets at the end of middle segment. Similar MT-doublet splitting is observed in human spermatozoa¹. Some CEM distal MT singlets fuse at the distal ciliary tip, a feature that has been not described before.

We found that CEM axonemal ultrastructure is modulated MT post-translational modifications. CCPP-1 is a tubulin deglutamylase that is required for B-tubule stability². We found that the TTL-11 glutamylase antagonizes CCPP-1 function in CEM neurons. In a ttl-11 mutant background, distal B-tubules in the CEM cilia are stabilized as nine doublets. Combined, our results indicated that A-B tubule spans in CEM cilia are modulated by glutamylation state.

Alpha tubulin-6 (tba-6) is required to establish MT ultrastructure in CEM cilia. By serial section TEM analysis of tba-6 mutants, we found that B-tubules in MT doublets fail to extend as separate singlets and terminate at the end of the middle segment. A-tubule singlets appeared unaffected. These results suggest that A- and B-tubule singlets are of different tubulin composition, and that B-tubules require tba-6.

Afzelius, B., Dallai, R., Lanzavecchia, S. & Bellon, P. Flagellar structure in normal human spermatozoa and in spermatozoa that lack dynein arms. *Tissue and Cell* 27, 241–247 (1995).

O'Hagan, R. et al. The Tubulin Deglutamylase CCPP-1 Regulates the Function and Stability of Sensory Cilia in *C. elegans*. *Current biology* 1–10 (2011). doi:10.1016/j.cub.2011.08.049

P989

Board Number: B315

The Pleiotropic Phenotypes of a Chlamydomonas Mutant Defective in a Flagellar Nucleoside Diphosphate Kinase.

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Large quantities of ATP and GTP are continually consumed in reactions central to various cilia and flagella functions, such as trafficking, tubulin turnover, signaling, protein folding and rhythmic beating. While mitochondria located near the flagella base could be the source of these nucleotides, flagella also harbor enzymes involved in NTP generation. To understand the metabolic schemes used to supply

these energy-requiring activities, we investigated a conserved nucleoside diphosphate kinase, NDK5, that is a constitutive subunit of the axonemal radial spoke complex in *Chlamydomonas* flagella. NDKs act to maintain the balance of nucleotide species by transferring γ -phosphate from copious NTP species to NDP of the scarcer ones. Curiously, while a mutant lacking the entire radial spoke has only paralyzed flagella, wild type transgenic strains expressing inactive NDK5_{H121A}-His that is incapable of phosphate transfer generate half-length, paralyzed flagella. A newly recovered *ndk5* mutant also generates paralyzed flagella, missing NDK5 and two additional radial spoke subunits - RSP1 in the head module and HSP40 that is thought to refold the head-neck region of the radial spoke precursor assembled in the cell body after it has been delivered to the flagellar tip. After deflagellation, the *ndk5* mutant regenerated shorter flagella and at a slower pace than the control. In minimal media lacking acetate that could be used for energy metabolism, flagellar length was dramatically reduced. Surprisingly, NDK5-deficient gametes also cannot mate. These data suggest that NDK5 is a critical member of an overlapping enzymatic network designed to couple nucleotide metabolism with radial spoke assembly and thus to meet the fluctuating energy demands of these complex organelles.

P990

Board Number: B316

An assay for clogging the ciliary pore complex distinguishes mechanisms of cytosolic and membrane protein entry.

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As a cellular organelle, the cilium contains a unique protein composition. Entry of both membrane and cytosolic components is tightly regulated by gating mechanisms at the cilium base, however, the mechanistic details of ciliary gating are largely unknown. We previously proposed that entry of cytosolic components is regulated by mechanisms similar to those of nuclear transport and is dependent on nucleoporins (NUPs) which comprise a ciliary pore complex (CPC). To investigate ciliary gating mechanisms, we developed a system to clog the pore by inhibiting NUP function via forced dimerization. We targeted NUP62, a component of the central channel of the nuclear pore complex (NPC), for forced dimerization by tagging it with the homodimerizing Fv domain. As proof of principle, we show that forced dimerization of NUP62-Fv attenuated active transport of bovine serum albumin into the nuclear compartment and of the kinesin-2 motor KIF17 into the ciliary compartment. Using the pore clogging technique, we find that forced dimerization of NUP62 attenuated the gated entry of cytosolic proteins but did not affect entry of membrane proteins or diffusional entry of small cytosolic proteins. We propose a model in which active transport of cytosolic proteins into both nuclear and ciliary compartments requires functional NUPs of the central pore whereas lateral entry of membrane proteins utilizes a different mechanism that is likely specific to each organelle's limiting membrane.

P991**Board Number: B317****PI3K/Akt signaling regulates ciliogenesis initiation via Rab11-effector vesicular trafficking switch.**V. Walia¹, C. Insinna (Kettenhofen)¹, Q. Lu¹, S. Specht¹, C.J. Westlake²;¹LCDS, National Cancer Institute-Frederick, Frederick, MD, ²National Cancer Institute-Frederick, Frederick, MD

Defects in primary cilium formation and signaling are associated with a growing list of genetic diseases and have been linked to certain cancers. A critical step in the initiation of ciliogenesis requires pre-ciliary vesicle trafficking to the mother centriole. Previously, we showed that serum starvation induced rapid association of Rabin8, a Rab8 GEF, with Rab11 pre-ciliary vesicles, a step needed to initiate ciliogenesis. We hypothesized that pre-ciliary trafficking depends on growth factor signaling. To test this hypothesis, we screened growth factors in RPE cells cultured in serum-free condition and identified lysophosphatidic acid (LPA) and TGF- β as novel inhibitors of ciliogenesis. Interestingly, only LPA blocked Rabin8 pre-ciliary trafficking suggesting that LPA functions specifically at the ciliogenesis initiation step. We show that the LPAR1 receptor, but not LPAR2-5, functions in the inhibition of pre-ciliary trafficking and ciliogenesis. Using chemical inhibitors against LPAR1 downstream pathways, we determined that inhibition of PI3K/Akt signaling induced Rabin8 pre-ciliary trafficking and promoted ciliogenesis, whereas other LPAR1 signaling pathways (Ras, Rac, or PLC) had no effect. Inhibition of PI3K/Akt signaling also promotes ciliogenesis in other cell lines suggesting that this pathway is a global regulator of cilia formation. To understand how Akt regulates pre-ciliary trafficking, we examined Akt-phosphorylation on Rab11 and Rabin8. While Rabin8 was found to be an Akt substrate, there was no observed effect of this site on Rab11 binding or ciliogenesis. We next theorized that Akt-phosphorylation regulates Rab11-Rabin8 interaction indirectly via a Rab-effector switch. RNAi studies for Rab11-effectors demonstrated that WDR44/Rabphilin-11 ablation promotes Rabin8 centrosomal trafficking in the presence of serum. WDR44 is the first reported Rab11 effector but its function is not well understood. We mapped Akt sites on WDR44 within the Rab11 binding domain and showed that phosphorylation status of WDR44 coincided with Rab11-Rabin8 interaction upon serum starvation. Importantly, a phospho-mimetic WDR44 mutant showed stronger binding to Rab11 and inhibited ciliation upon serum starvation. Together these findings uncover a novel role for a growth factor signaling network mediated by Akt kinase and its downstream substrate, WDR44, in the regulation of pre-ciliary vesicle trafficking and ciliogenesis initiation.

P992**Board Number: B318****Cryo-electron tomography reveals the 3D structure of the ciliary tip.**A.L. Mleczak¹, P. Kiesel¹, J. Mahamid², O. Filina¹, L. Stepanek¹, G. Marini¹, G. Pigino¹;¹Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany, ²Max Planck Institute of Biochemistry, Martinsried, Germany

Eukaryotic cilia are microtubule-based organelles that allow cells to move and sense extracellular signals. The way the cell assembles the cilium and its complex axonemal structure is not completely understood. It is known that building blocks of the axoneme are transported by the intraflagellar transport trains at the tip of the cilium (1) where the assembly takes place (2). However, the mechanism and the molecular machinery that regulates this process are largely unknown. William Dentler and collaborators observed typical capping structures at the plus end of axonemal microtubules in a number of species (3). These structures might be involved in the regulation of axonemal assembly and disassembly.

Here we used cryo-electron tomography to reveal the 3D architecture of the ciliary tip in the free-living protozoan *Tetrahymena thermophila*. We confirm the presence of ciliary capping structures in membranated cilia that are preserved in a near-native, hydrated state, typical of the cryo-vitrification technique. Also, for the first time we show the 3D arrangement of these structures. A Central Microtubule Cap (CMC), which terminates the Central Pair of Microtubules (CP MTs) consists of two plugs inserted into the lumen of CP MTs, a density between the CP MTs, a plate located perpendicularly to CP MTs, and a bead at the very end of CMC. This globular structure is tightly connected to the ciliary membrane. Distal Filaments (DFs) are visible at the plus ends of A-tubules and also connect with the membrane. The B-tubules do not present any capping structure and terminate proximally to the A-tubules forming the Singlet Zone. Because most of the microtubule associated proteins are not present in the singlet zone, the axoneme at the tip shows much lower complexity than the main part of cilium. We are now using a computational method called sub-tomogram averaging to reconstruct high-resolution models of the microtubule capping structures of the ciliary tip.

Literature:

1. Kozminski KG, Beech PL, and Rosenbaum JL. 1995. "The *Chlamydomonas* Kinesin-Like Protein FLA10 Is Involved in Motility Associated with the Flagellar Membrane." *The Journal of Biophysical and Biochemical Cytology* 131: 1517–1527.
2. Johnson KA, and Rosenbaum JL. 1992. "Polarity of Flagellar Assembly in *Chlamydomonas*." *The Journal of Cell Biology* 119: 1605–1611.
3. Dentler WL, and Rosenbaum JL. 1977. "Flagellar Elongation and Shortening in *Chlamydomonas* III. Structures Attached to the Tips of Flagellar Microtubules and Their Relationship to the Directionality of Flagellar Microtubule Assembly." *The Journal of Cell Biology* 74: 747–759.

P993**Board Number: B319****EHD proteins coordinate membrane reorganization and fusion to initiate early steps of ciliogenesis.**

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The primary cilium is a membrane-bound, microtubule based sensory organelle that plays essential roles in development and disease pathways. Cilia biogenesis requires coordination of a series of processes including, mother centriole to basal body transformation, recruitment of intraflagellar transport (IFT) and transition zone (TZ) proteins, and axoneme formation and association with a developing ciliary membrane. Membrane association with the distal appendages of the mother centriole is a critical step in ciliogenesis initiation. The membrane trafficking Rab GTPase Rab11-Rab8 cascade plays key roles in early ciliary membrane assembly, but the molecular details remain unclear. Here, we show that the membrane shaping proteins EHD1 and EHD3, in association with the Rab11-Rab8 ciliogenesis cascade, function in cilia assembly in zebrafish and mammalian cells. We discovered that EHD proteins localize to Rab11 pre-ciliary vesicles that dock to the distal appendages of the mother centriole, membranes we refer to as distal appendage vesicles (DAV). Using live and super-resolution imaging, as well as electron microscopy approaches, we established that EHD proteins are essential for the formation of the larger pre-axonemal ciliary vesicle (CV) from DAVs. Furthermore, we show that EHD1-dependent CV formation is critical for initiating mother centriole to basal body transformation and recruitment of IFT20 and transition zone proteins. Surprisingly, we found that Rab8 is recruited for ciliary membrane growth only after these steps, and in coordination with axonemal assembly. Investigations into the molecular mechanism of these early ciliogenesis initiation steps suggested that EHD proteins tubulate DAVs, bringing them in close proximity to allow fusion into the CV. This step is required for CP110 removal from the distal end of the mother centriole prior to recruitment of IFT20 and TZ proteins. Based on these findings we predicted that SNAREs, regulators of membrane fusion, would be important for CV assembly and ciliogenesis progression. We show that the SNARE SNAP29, an EHD1 interacting protein, is required for ciliogenesis and localizes with EHD1/3 on early ciliary membrane structures. Together, our studies provide new molecular mechanisms informing the classically described intracellular ciliogenesis pathway and uncover a previously uncharacterized step in ciliary assembly.

P994**Board Number: B320****Actin is required for IFT regulation in *Chlamydomonas reinhardtii*.**P. Avasthi¹, M. Onishi², J. Karpiak³, R. Yamamoto⁴, L. Mackinder⁵, M.C. Jonikas⁵, W.S. Sale⁴, B. Shoichet³, J. Pringle², W. Marshall⁶;¹Biochemistry and Biophysics, University of California, San Francisco, San Francisco, CA, ²Genetics, Stanford Univ Med Ctr, Stanford, CA, ³Pharmaceutical Chemistry, University of California, San Francisco, San Francisco, CA, ⁴Department of Cell Biology, Emory University School of Medicine, Atlanta, GA, ⁵Plant Biology, Carnegie Institution for Science, Stanford, CA, ⁶Biochemistry and Biophysics Dept., University of California, San Francisco, San Francisco, CA

Assembly of cilia/flagella require the bidirectional transport of motors and cargo, termed intraflagellar transport (IFT). Recruitment of IFT machinery to the base of flagella is increased in shorter, rapidly assembling flagella and is reduced at the base of longer, slowly assembling flagella that are approaching their final steady state length. Disruption of the actin network has been shown to affect ciliary formation, length and number but the mechanisms are unknown. We utilized the green alga *Chlamydomonas reinhardtii* to investigate potential mechanisms by which actin might regulate cilia/flagella formation. In *Chlamydomonas*, actin is found in the inner dynein complexes within flagella as well as in the cell body. While, previous attempts to localize filamentous actin in vegetative cells have failed, we have localized filaments to both perinuclear and anterior regions using expression of a tagged filament binding peptide, Lifeact-Venus. We also show that actin disruption using small molecule inhibitors and a null actin mutant results in impaired flagellar regeneration, IFT injection and recruitment of IFT material to the flagellar base, with recruitment lacking the normal dependence on flagellar length. Each phenotype is recapitulated when using a myosin II inhibitor and we have modeled the potential binding of this inhibitor to a *Chlamydomonas* myosin. Mid-cell localization of a tagged version of this myosin is disrupted when flagellar regeneration is induced. Our data suggest actin and myosin are involved in establishing the length dependence of flagellar assembly via IFT regulation.

P995**Board Number: B321****Rsg1 Is Required for Normal Cilia Number, Limb Patterning, and Heart Development in the Mouse Embryo.**S.O. Agbu¹, K.V. Anderson¹;¹Developmental Biology Program, Sloan-Kettering Institute, New York, NY

We use forward genetic screens to uncover novel regulators of murine embryonic development. From a recent screen, we identified the *pixiebob* (*pxb*) mutant line based on abnormal limb development. This line carries a recessive mutation that results in lethality at embryonic day 12.5 (e12.5). Recombination mapping and deep sequencing showed that the *pixiebob* mutation is a thymine-to-adenine transversion in the *Rsg1* gene, which results in a valine-to-aspartic acid substitution. *Rsg1* is a member of the

Rem2/Rab family of small GTPases that was identified based on its interaction with Fuzzy, a protein required for cilia formation in vertebrates. Analysis of *Rsg1* morphants showed that it plays a role in the formation of motile cilia in the multiciliated cells of the *Xenopus laevis* epithelium, where it appears to facilitate the trafficking of proteins required for ciliogenesis. In mouse embryonic fibroblasts derived from *pxb* embryos, the number of cells with primary cilia is reduced to about 20% of wild-type levels. At e11.5, *pxb* embryos exhibit preaxial polydactyly and edema, which implies that the *pxb* mutation interferes with limb and heart development. An increase in *Gremlin1* and *Fgf8* expression is observed in *pxb* limbs by RNA in-situ hybridization, suggesting that the *pxb* mutation affects signaling pathways that are essential for anterior-posterior limb patterning. Primary cilia are important for Hedgehog signaling, and we find that Gli3, which negatively regulates *Gremlin1* and *Fgf8*, is reduced upon Western blot analysis in *pxb* embryos, consistent with a disruption in cilia-dependent Hedgehog signaling. Hematoxylin and eosin (H&E) staining of *pxb* heart tissue reveals a cardiac septal defect, which may be responsible for the embryonic lethality observed. Our studies will define the function for *Rsg1* in primary cilia formation and in mammalian embryonic development.

P996

Board Number: B322

Disa1p-dependent kinetodesmal fiber elongation is required to organize *Tetrahymena* ciliary arrays.

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Motile cilia are whip-like projections that generate hydrodynamic force. Motile cilia function within ciliary arrays that contain hundreds of cilia positioned with a high degree of spatial organization. Ciliary arrays are required for symmetry breaking during embryogenesis, fluid flow within complex tissues and the directed movement of unicellular organisms. Cilia are nucleated by basal bodies, which are microtubule scaffolds that are rotationally polarized due to the asymmetric attachment of auxiliary structures. Since basal body polarity dictates the direction of ciliary beating, basal body polarity must be aligned along a common axis of cellular polarity to generate coherent cilia-generated fluid flow. However, the structures and molecules that align basal body polarity remain ill-defined. Here, we investigate the mechanisms that establish and maintain basal body polarity in the multi-ciliated unicellular organism *Tetrahymena*. We accomplish this by analyzing the classical mutant *disorganizedA* (*disa1*) that exhibits defective basal body organization and rotational polarity. Using next-generation sequencing, we show that *disa1* results from a single nucleotide substitution that causes a loss-of-function of a novel 31 kDa coiled-coil protein that we name Disa1p. Disa1p localizes to the proximal

portion of the anterior face of the basal body at an asymmetric structure called the kinetodesmal fiber (KF). KFs are an evolutionarily conserved class of striated fibers that attach asymmetrically to basal bodies and project in a planar fashion directly opposite the ciliary power stroke. We show that KFs are dynamic structures that elongate in response to increased cilia-generated force. In the absence of functional *Disa1p*, KFs are unable to elongate, causing severe KF truncation. KFs structurally oppose the ciliary power stroke and terminate adjacent to neighboring basal bodies. It has been proposed that KFs provide a physical linkage that maintains basal body polarity by stabilizing basal bodies against ciliary forces. We test this hypothesis using a variety of manipulations to modulate ciliary force in *disa1* cells. We demonstrate that short KFs allow basal bodies to rotate at the cell cortex in response to increased ciliary forces, which randomizes KF orientation relative to cellular polarity. Conversely, randomization of basal body polarity is rescued by reducing ciliary force. Finally, we show that nascent basal bodies traverse the KF as they migrate towards their eventual position at the cell cortex. Therefore, the truncated and misoriented KFs that result from *Disa1p* mutation propagate basal body disorganization, demonstrating that *Disa1* is a genic input into the structural inheritance of basal body polarity and organization in *Tetrahymena*.

P997

Board Number: B323

The uncovering of three new *Chlamydomonas* deflagellation genes and the identification of ADF1.

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The two flagella of *Chlamydomonas reinhardtii* are shed upon exposure to cellular stresses, such as pH shock. In response to intracellular acidification, a Ca²⁺-influx occurs that ultimately activates severing of the axonemal microtubules at a site distal to the flagellar transition zone. Our previous screen for deflagellation-defective mutants produced multiple alleles of FA1 and FA2, which are defective in axonemal severing, and multiple alleles of ADF1, which are defective in acid-induced Ca²⁺-influx. Both FA1 and FA2 were successfully cloned and encode a scaffolding protein and a NIMA-related kinase, respectively, but the identity of ADF1 was elusive, until now. Additionally, several predicted players in the deflagellation pathway were not uncovered in the original screen. We expected, but did not find fa mutants defective in a Ca²⁺-sensor or in a microtubule severing protein. Armed with advances in whole genome sequencing (WGS) and strategies to rapidly identify point mutants, we undertook a new screen of UV-mutagenized cells. We recovered 29 new mutant strains, including two new alleles of FA1, four new alleles of FA2, fourteen new alleles of ADF1, and alleles of three new deflagellation genes, including two temperature-sensitive alleles of FA3, one (ts) allele of ADF2, one allele of ADF3 and one allele of ADF4, which exhibits a novel gamete-specific deflagellation-defective phenotype. We had previously mapped ADF1 to a 450 kb region of chromosome 9, and WGS of three new alleles and one of the original alleles of ADF1 revealed that all four mutant alleles carry mutations in a TRP channel that lies at the heart of our mapped region, consistent with our prediction of a calcium-permeant channel in the

acid deflagellation pathway. We have rescued the phenotype and identify TRP15 as the channel that mediates calcium influx in response to intracellular acidification.

P998

Board Number: B324

A Genetic Screen for Molecules that Regulate Axoneme Stability in Response to Post-Translational Microtubule Glutamylation.

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When polymerized into microtubules (MTs), tubulins accumulate post-translational modifications (PTMs) such as polyglutamylation. PTMs have been proposed to act as signposts that regulate MT stability and the activities of other proteins that bind MTs, such as kinesin and dynein motors. The MTs that form ciliary axonemes are especially prone to post-translational modification, but the roles that MT PTMs play in cilia are largely unknown.

The enzymes that regulate post-translational glutamylation of tubulins profoundly affect cilia structure in *C. elegans* sensory neurons. Loss of CCPP-1, a homolog of murine Ccp1, which deglutamylates MTs, causes the progressive degeneration of amphid neuronal cilia. However, ciliary degeneration in *ccpp-1* mutants can be suppressed by deletion of *ttll-4*, *ttll-5*, or *ttll-11*, which encode glutamylases that add glutamylation to MTs. These data suggest that excess MT glutamylation destabilizes ciliary MTs, leading to ciliary degeneration.

To understand the mechanism by which MT glutamylation regulates ciliary MT stability, we performed a forward genetic screen for suppressors of *ccpp-1*-induced amphid degeneration. To visualize intact versus degenerate cilia, we soaked animals in a fluorescent dye that is absorbed through cilia and illuminates intact amphid cilia. In *ccpp-1* mutants, the ciliary degeneration of amphid neurons is usually complete by adulthood, such that adults fail to take up the dye and appear completely "dark." Therefore, we searched for suppressor mutations by dye-filling the adult F2 progeny of mutagenized *ccpp-1* animals and looking for animals with amphids that were illuminated by the dye.

We isolated fifteen suppressor mutants, including some that fail to complement *ttll-4*, *ttll-5*, and *ttll-11* mutations. We hope that our suppressor alleles will illuminate pathways by which post-translational glutamylation regulates MT structure and function.

P999**Board Number: B325****HDAC2 mediates loss of primary cilia in pancreatic ductal adenocarcinoma cells.**T. Kobayashi^{1,2}, K. Nakazono¹, M. Tokuda¹, B.D. Dynlacht², H. Itoh^{1,2};¹Nara Institute of Science and Technology, Ikoma, Japan, ²School of Medicine, New York University, New York, NY

Primary cilium functions as a cellular antenna to sense extracellular environment and transduces signals to the cell. Loss of primary cilia is frequently observed in tumor cells, including pancreatic ductal adenocarcinoma (PDAC) cells, suggesting that the absence of this organelle may promote tumorigenesis through aberrant signal transduction and the inability to exit the cell cycle. However, the molecular mechanisms that explain how PDAC cells lose primary cilia remain unknown. We found that treatment with several histone deacetylase (HDAC) inhibitors restores primary cilia in PDAC cells. We next performed knock-down experiments using siRNAs and observed that silencing of HDAC2, a known transcriptional regulator, induces primary cilia formation. On the other hand, inhibition or depletion of HDAC6, which was previously reported to be required for cilia resorption, had no effect on the number of primary cilia. These data suggest that HDAC2 specifically suppresses primary cilia formation in PDAC cells. The mechanistic and practical implications of these studies will be discussed.

Sensory and Signaling Functions of Cilia**P1000****Board Number: B326****Ciliary membrane protein trafficking in *Chlamydomonas* is uni-directional and depends on cytoplasmic retrograde IFT motor activity and shedding of ciliary ectosomes.**J. Ning¹, M. Cao¹, C. Hernandez-Lara¹, O. Belzile¹, Q. Wang¹, Y. Liu¹, W.J. Snell¹;¹Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX

The role of the primary cilium in key signaling pathways depends on dynamic regulation of ciliary membrane protein composition, yet we know little about the motors or membrane events that regulate ciliary membrane protein trafficking in fully formed organelles. Previously we showed that cilium-generated signaling during ciliary adhesion between plus and minus gametes of *Chlamydomonas* induced rapid, cytoplasmic microtubule-dependent redistribution of the membrane polypeptide, SAG1-C65, from the plasma membrane to the periciliary region and the ciliary membrane, and that protein redistribution depended on cytoplasmic microtubules. Here, we report that the retrograde IFT motor, cytoplasmic dynein 1b, is required in the cytoplasm for this rapid redistribution. Furthermore, we find that trafficking of SAG1-C65 into cilia is unidirectional. During adhesion and signaling, SAG1-C65-HA is depleted from cells and released into the medium as ciliary ectosomes. Shedding of SAG1-C65 is the

major fate of the protein and the entire complement of pre-existing cellular SAG1-C65 is shed during sustained adhesion/signaling in the form of a distinct ciliary ectosome membrane compartment that retains signal-inducing activity. Thus, during ciliary signaling, ciliary trafficking of a membrane protein is uni-directional and cells regulate ciliary membrane protein composition through cytoplasmic action of the retrograde IFT motor and shedding of ciliary ectosomes. Supported by NIH GM25661.

P1001

Board Number: B327

The Intraflagellar Transport Protein IFT27/RABL4 Promotes BBSome Exit from Cilia through the GTPase ARL6/BBS3.

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The sorting of signaling receptors into and out of cilia relies on the BBSome, a complex of Bardet-Biedl syndrome (BBS) proteins, and on the intraflagellar transport (IFT) machinery. GTP loading onto the Arf-like GTPase ARL6/BBS3 drives assembly of a membrane-apposed BBSome coat that promotes cargo entry into cilia, yet how and where ARL6 is activated remains elusive. Here, we show that the Rab-like GTPase IFT27/RABL4, a known component of IFT complex B, promotes the exit of BBSome and associated cargoes from cilia. Unbiased proteomics and biochemical reconstitution assays show that, upon disengagement from the rest of IFT-B, IFT27 directly interacts with the nucleotide-free form of ARL6. Furthermore, IFT27 prevents aggregation of nucleotide-free ARL6 in solution. Thus, we propose that IFT27 separates from IFT-B inside cilia to promote ARL6 activation, BBSome coat assembly and subsequent ciliary exit, mirroring the process by which BBSome mediates cargo entry into cilia.

P1002

Board Number: B328

IFT27 Links the BBSome to IFT for Maintenance of Ciliary Signaling Compartment.

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During the past decade, many studies shed light on cilia as sensory and motile organelles which play major roles in vertebrate development. Indeed, defects in cilia formation lead to a wide range of

structural birth defects. To build all mammalian cilia, the intraflagellar transport (IFT) machinery is required. Proteins of both retrograde and anterograde IFT complexes are highly conserved across ciliated species from *Chlamydomonas* to human. However, two proteins of the core IFT complex B - IFT25 and IFT27 - are absent from certain ciliated organisms like *Caenorhabditis* and *Drosophila* suggesting that they may have a unique role distinct from ciliogenesis. To get better insight in their role in mammal development, we generated *Ift25* and *Ift27* null mice. Mutant mice die at birth with major developmental defects indicative of Hedgehog signaling dysfunction. Since Hedgehog signaling occurs within cilia in mammals, we looked for cilia dysfunction at the cellular level and show that mutant cells are able to grow cilia. However, cilia lacking IFT25/27 have defects in the signal-dependent transport of multiple Hedgehog components resulting in the accumulation of patched-1 and smoothed. Similarly smoothed accumulates in cilia on cells mutated for BBSome components or the BBS binding protein/regulator *Lztf1*. Interestingly, the BBSome and *Lztf1* accumulate to high levels in *Ift27* mutant cilia as well. Since *Lztf1* mutant cells accumulate BBSome but not IFT27 it is likely that *Lztf1* functions downstream of IFT27 to couple the BBSome to the IFT particle for coordinated removal of patched-1 and smoothed from cilia during hedgehog signaling.

P1003

Board Number: B329

The signaling scaffold protein Parafusin localizes to both cilia and the nucleus.

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Signaling scaffold proteins could play a role in eukaryotic cell evolution. One of these proteins is parafusin (PFUS), a component of dense core secretory vesicle (DSCV) scaffolds in apicomplexan exocytosis. PFUS, a 63 kDa cytoplasmic protein, first discovered in the unicellular eukaryote *Paramecium* is closely related to eubacterial rather than archeal phosphoglucomutases (PGM) but has no PGM activity. A PFUS specific peptide antibody was made to an amino acid sequence not present in PGM. Immunofluorescence microscopy with this antibody showed that PFUS was localized around the base of the primary cilia in a variety of mammalian cultured cells, including mouse embryonic (MEFs) and human foreskin fibroblasts (hFFs), human carcinoma stem cells (NT-2 cells), and human retinal pigment epithelial (RPE) cells. PFUS also localized to the nucleus of fibroblasts, and particularly to nucleoli of MEFs. Localization studies were confirmed by Western blot analysis, showing that the antibody specifically recognized PFUS in both MEF cytoplasmic and nuclear fractions and that the single 63kDa band in the blots disappeared when the fractions were treated with blocking peptide. Finally, immunofluorescence microscopy showed that PFUS localized to nuclei and cilia as well as DSCVs in *Paramecium*. These results support the possibility that PFUS could traffic between nucleus and cilia, as is true for many molecules involved in signaling pathways. It seems likely that the cilium and the nucleus both evolved around the time of eukaryotic emergence. We hypothesize that near the beginnings of eukaryotic cell evolution, scaffold proteins such as PFUS arose as peripheral membrane protein

identifiers and signaling molecules for cytoplasmic membrane trafficking and were employed similarly during the subsequent evolution of exocytic, nuclear transport, and ciliogenic mechanisms.

P1004

Board Number: B330

Superresolution Pattern Recognition Reveals an Architectural Map of the Ciliary Transition Zone.

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The ciliary transition zone (TZ), which is part of the gating apparatus at the ciliary base, is home to a large number of ciliopathy molecules. The architecture of the TZ, however, is poorly understood, hindering mechanistic understanding of its role as a diffusion barrier. Taking advantage of special patterns resulting from convolution of the slender point spread function (PSF) of stimulated emission depletion (STED) superresolution microscopy and ring-shaped protein distributions, we were able to reveal differential localization of TZ proteins. Using a series of these STED images mapped to electron microscopy (EM) images, we defined novel geometric distributions of TCTN2, TMEM67, MKS1, and RPGRIP1L from the ciliary membrane to the axoneme at the same axial level. CEP290 is surprisingly localized at a different axial level toward the proximal side of the TZ. Upon this molecular architecture, two reservoirs of intraflagellar transport (IFT) particles, correlating with phases of ciliary growth, are present: one colocalized with the transition fibers while the other situated beyond the distal edge of the TZ. Taken together, we have pushed the limit of subdiffraction imaging to localize multiple key proteins at the ciliary base, revealing an unprecedented structural framework and transport variations upon this framework.

P1005

Board Number: B331

Primary cilia's role in cellular gravitransduction.

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Gravity is one of the most fundamental forces which organisms have to cope with on Earth, and inducing changes in the magnitude of this force leads to various cellular responses including cell proliferation, migration, and differentiation. Although little is known about how cells perceive gravitational acceleration and trigger biochemical alterations upon changes in gravity force, hypotheses include

primary cilia, non-motile cellular antennae, acting as gravity transducers due to their ability to sense various mechanical stimuli. However, strong empirical evidence to support this idea has not been established yet. In our study, we show that primary cilia in MC3T3-E1 osteoblast cells are required for the mechanotransduction of hyper-acceleration stimulus into proliferative responses. We also report that the translation of hyper-acceleration into cellular response, through primary cilia, involves regulating beta-catenin levels and contact inhibition of cell growth. Our results demonstrate that primary cilia in MC3T3-E1 cells have a role in mediating cellular gravitansduction. The existence of such cellular transduction element suggests that the gravity force, though nearly constant on Earth, might be a regulatory factor for cell fate determination. Furthermore, we expect our study to be a beginning for more sophisticated research investigating the mechanisms with which organisms perceive gravitational acceleration. This might lead to a better understanding of bone disorders, e.g. bone loss, particularly in the context of space research and medicine.

P1006

Board Number: B332

Tuning cellular mechanosensitivity: Mechanical and chemical modulation of primary cilia bending.

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The primary cilium is a solitary, nonmotile microtubule-based protrusion found on nearly every cell in the human body and has been established as a mechanosensor across several tissues. Several groups have reported cilia length adapts in response to mechanical stimuli while other groups have reported relatively small changes in length can affect cilia deflection and downstream load-induced changes in gene and protein expression. Collectively, this suggests cilia mechanosensitivity may be modulated. Our lab has shown that when a cilium is exposed to fluid flow and flow is stopped, the cilium often does not return to its original position. We hypothesized that exposure to flow increases the cilium's bending stiffness. Mouse inner medullary collecting duct cells transfected with somatostatin 3 receptor fused to GFP were exposed to a wall shear stress of 0.25 Pa for two periods of flow separated by a rest period. Cilia bending behavior was captured with a confocal microscope and modeled as a beam anchored by a torsional spring. We found a significant increase in bending stiffness along the ciliary axoneme and torsional stiffness at the base before and after the rest period, an increase of 2.2 ± 0.4 and 4.0 ± 0.8 fold ($n=12$), respectively. We hypothesized that this stiffening may be mimicked by altering tubulin. Acetylation and other post-translational modifications of tubulin have previously been shown to increase microtubule stiffness and interestingly, microtubule acetylation increases with load. To increase acetylation, we inhibited deacetylation with two different methods: tubacin, a chemical inhibitor of microtubule-associated histone deacetylase 6 (HDAC6), and HDAC6 siRNA. We found a similar stiffening effect in cilium bending, a 4.0 ± 1.3 fold increase with tubacin and a 2.7 ± 0.9 fold increase with HDAC6 knockdown ($n=5$ /group), and hypothesized that the stiffening of the cilium would lead to decreased mechanosensitivity. We exposed cells to oscillatory fluid flow (1 hr, 1 Pa peak shear

stress) and measured gene expression. We confirmed downregulation of HDAC6 (30.5±8.9% decrease in HDAC6/GAPDH expression, n=10/group) and increased acetylation (marked increase in acetylated α tubulin staining). This increase in acetylation inhibited flow-induced increases in cyclooxygenase-2/GAPDH expression by 55.9±16.3% (n=5/group). Using a transgenic mouse expressing GFP in primary cilia, allowing cilia to be easily visualized *in situ*, we measured *in vivo* primary cilia bending in the kidney proximal tubules of a live mouse and validated previous *in vitro* measurements made by our lab and others. Taken together, our data demonstrate that the cilia bending response may be modulated by both mechanical and chemical stimuli and is coupled to cellular mechanosensitivity.

P1007

Board Number: B333

Polycystins have discrete patterns of expression in Hydra and may regulate Hydra behaviors.

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The cnidarian *Hydra* is one of the most primitive metazoans with a nervous system, but nevertheless displays complex behaviors, including cnidocyte (stinging cell) release and a stereotypical set of movements during its feeding response. Chemical, mechanical and photo stimuli have been shown to affect these behaviors, but the molecular mechanisms regulating these activities are still poorly understood. The purpose of this study is to determine if TRP (transient receptor potential) channels are expressed in hydra, and if they play a role in hydra behaviors.

TRP channels are a diverse superfamily of nonselective cation channels that function as polymodal cell sensors. A search of the hydra genome revealed the presence of multiple TRP subclasses, including several members of the TRP polycystin 2 (PC2) subfamily. Several potential polycystin 1 (PC1) homologs were also identified in the hydra genome. PC1 proteins are not TRP channels, but are known to interact with PC2 proteins; it has been proposed that the PC1 protein responds to a signal (chemical or mechanical) through its extracellular N terminus, and in turn gates its associated PC2 protein.

Whole mount *in situ* hybridizations revealed that three polycystin genes exhibit specific patterns of expression in hydra. H6908 (PC2) and H5453 (PC1) are strongly expressed in the tentacles (where most mature cnidocytes are located) and the basal disc. H3133 (PC2) is expressed in cells immediately surrounding the mouth, as well as in discrete patches of cells in the hydra body. To assess the possible role of these hydra polycystins, non-specific chemical inhibitors of PC2 channels were used in prey capture and mouth opening assays. The inhibitors reduced the ability of hydra to capture prey and also reduced mouth opening in response to glutathione (a known mouth-opening stimulus).

Examining the role of polycystins in hydra will lead to a further understanding of how signal transduction in single cells can lead to the development and evolution of complex behaviors. In addition, studying

polycystins in hydra can further our understanding of the function of these proteins in more complex organisms.

P1008

Board Number: B334

The Biogenesis Of Lysosome-Related Organelles Complex-1 (BLOC-1) Subunit Pallidin And The Exocyst Subunit Exo70 Are Involved In Membrane Protein Trafficking To The Primary Cilium.

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Most vertebrate cell types possess a single non-motile primary cilium on their cell surface. This organelle is thought to perceive the extracellular environment through the utilization of signaling receptors localized in the ciliary membrane and communicate these signals to the cell body. Currently, little is known on how these signaling receptors are targeted to this organelle. Primary cilia are assembled and maintained by the process of intraflagellar transport (IFT). This system is dependent on two protein complexes: IFT complex A and B. The complex B subunit IFT20 is the only IFT protein known to localize at both the primary cilium and Golgi apparatus. Previously we showed that IFT20 interacts with the golgin GMAP210. Furthermore, knockdown of IFT20 or knock out of GMAP210 results in reduced steady state levels of the membrane protein polycystin-2 in primary cilia. This has led to the proposal that both IFT20 and GMAP210 are important for sorting and/or transport of ciliary membrane proteins. However, the step(s) at which they function remains to be defined. Published large-scale yeast-two hybrid screens have identified new potential IFT20-interacting proteins. Two proteins of interest include the biogenesis of lysosome-related organelles complex-1 (BLOC-1) subunit pallidin and the exocyst subunit Exo70. We find that both pallidin and Exo70 co-immunoprecipitate with IFT20. Additionally, overexpression of either pallidin or Exo70 in IMCD cells displaces IFT20 from the Golgi apparatus. This evidence suggests that pallidin and Exo70 interact with IFT20. To further understand the role of IFT20 in trafficking ciliary membrane proteins, we developed a quantitative fluorescence-based pulse-chase assay to measure the time that it takes for membrane proteins to exit the Golgi apparatus and be delivered to the cilium. The shRNA knockdown of IFT20 or GMAP210 significantly decreases the rate of fibrocystin and polycystin-2 delivery to the cilium while the delivery rate of Smoothed is only modestly affected. The shRNA knockdown of Exo70 significantly decreases the rate of fibrocystin and polycystin-2 delivery to the cilium while the delivery rate of Smoothed is not affected. Lastly, the shRNA knockdown of pallidin significantly decreases the rate of polycystin-2 trafficking to the primary cilium. Ongoing research is focused on determining the mechanistic role of IFT20 and binding partners such as pallidin and Exo70 in ciliary membrane protein trafficking.

P1009**Board Number: B335****Role of the G-protein-coupled receptor, Gpr161, in vertebrate hedgehog signaling.**

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Long ignored as vestigial, primary cilia have now been established as sensory antennae in most vertebrate cells. Although, mechanisms driving formation of cilia have been extensively studied, the principles underlying signaling are largely unknown. The primary cilium is fundamentally important during developmental sonic hedgehog (Shh) signaling in vertebrates by acting as a compartment for dynamic localization of pathway components, and finally, regulating the bifunctional Gli transcription factors. The cAMP activated protein kinase A (PKA) triggers partial proteolytic processing that generates Gli repressors in a cilia-dependent manner; however, pathways that promote PKA activation, and the exact function of cilia is unclear. We previously showed that the tubby family proteins Tulp3 and Tubby, serve as an anchor between ciliary IFT-A complex and phosphatidylinositol 4,5-bisphosphate-rich membrane compartment in gating GPCRs to the cilia (Genes & Dev. 2010. 24: 2180-2193). We have recently identified an orphan ciliary GPCR, Gpr161 that acts as a negative regulator of Shh signaling during neural tube development by mediating the generation of Gli repressor via cAMP signaling. Active Shh signaling also results in removal of this receptor from the primary cilia (Cell 2013. 152: 210-223). Thus, Gpr161 is a candidate GPCR for activating PKA by generating a ciliary cAMP gradient, while also being regulated by Shh signaling in a positive feedback circuit. We are currently utilizing proteomic, cell biological and reverse genetic approaches to study distinct aspects of Gpr161 function, such as signaling, ciliary localization, and removal from cilia, and mechanistically investigating signaling in the context of intact cilia by uncoupling these functions. Dissecting mechanisms governing the regulation and signaling of Gpr161 in both cellular and organismal contexts, and extending these emerging principles to ciliary signaling, will reveal important insights into the role of cilia as signaling compartments.

P1010**Board Number: B336****EFHC1/Myoclonin regulates Wnt signaling, gene expression, cilia formation and neural plate and neural crest behavior in Xenopus.**

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Eukaryotic cilia are complex cellular structures known to be involved in a range of functions including fluid flow generation, cellular organization and signaling transduction during development. EFHC1 is a conserved ciliary component, with three conserved "DM10" domains, of unknown function and two

adjacent EF-hand, putative Ca²⁺ binding motifs in its C-terminal domain. Homologs have been identified in *Chlamydomonas*, *Tetrahymena*, and throughout vertebrates. Mutations in *EFHC1/Myoclonin* cause a dominant form of Juvenile Myoclonic Epilepsy (JME) in humans. Despite some description of EFHC1 expression, localization, and interactions, the exact cellular functions of the EFHC1 protein and why when it is defective it gives rise to the JME phenotype remain obscure. Here we used the ciliate *Tetrahymena* and clawed frog *Xenopus laevis* to explore the cellular and developmental functions of EFHC1. Unlike *Tetrahymena* and mammals that have two EFHC-like proteins, there appears to be only one EFHC1 gene in *X. laevis*. *X.l.*EFHC1 RNA is supplied maternally and increases dramatically at the onset of gastrulation. *In situ* hybridization analysis reveals EFHC1 expression in the multiciliated cells of the epidermis and later in the developing central nervous system, the eye, the otic vesicle, and the neural crest. The morpholino-mediated down regulation of EFHC1 in *Xenopus* embryos produces defects in ciliated cell and cilia formation, the patterning of the neural plate and the formation and migration of the neural crest. These effects can be rescued by an EFHC1-GFP chimera that localizes to the axonemal region of cilia in multiciliated epidermal cells. The *Tetrahymena* proteins exhibit similar localization and deletion of either gene produces little phenotype. A double mutant strain is being constructed. Reduction of *Xenopus* EFHC1 leads to a Wnt-mediated feed forward loop that up-regulates Wnt8 RNA and down-regulations in *Tubb2b* (neuro-tubulin) RNA. Many but not all EFHC1 morphant phenotypes are rescued by blocking Wnt signaling using injection of SFRP2 RNA. Mutational studies indicate that the N-terminal domain and the first DM10 domain are required for full phenotypic rescue. The same domains of the *Tetrahymena* protein are required for axoneme and basal body localization. Finally, *X.l.*EFHC1 over-expression induces the ectopic expression of the bHLH transcription factor Twist1. Together, these observations identify a previously unsuspected role of EFHC1 in the regulation of Wnt signaling, and suggest that ciliary function and Wnt/Twist1 gene expression might be relevant to the etiology of JME.

P1011

Board Number: B337

Canonical Wnt signaling pathway regulates not only the odontoblast differentiation through primary cilia but also formation of primary cilia.

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Despite the facts that primary cilium appear ubiquitously in human body, very little is known about its physiological functions. Odontoblasts, which serve for dentin formation, also possess the primary cilia *in situ*. However, their physiological functions remain to be investigated.

To examine the roles of primary cilium in odontoblasts, we employed RNAi strategy to knockdown *Intraflagellar transport protein-88 (Ift88)*, which is required for the ciliogenesis in most of the quiescent cells, on cultured odontoblastic KN-3 cells. As a result, the ratio of cells positive for cilia has been decreased. Under this condition, KN-3 cells showed significantly retarded deposition of minerals on the

substrate of culture well. On the contrary, the production of dentin sialophosphoprotein (DSPP), a molecular marker of odontoblast differentiation, was promoted. Since *Ift88* knockdown resulted in increased accumulation of β -catenin, we considered that IFT88 modulates the odontoblastic differentiation through canonical Wnt signaling pathway. In order to test this hypothesis, we stimulate the canonical Wnt signaling pathway by recombinant Wnt3a, a representative agonist for canonical Wnt signaling pathway, or SB216763, glycogen synthase kinase-3 β inhibitor. Both treatments increased DSPP production and suppressed alkaline phosphatase (ALP) activity, the phenomena recapitulating the effects of IFT88 suppression on KN-3 cells. Moreover, ALP activity was gradually increased by progression of ciliogenesis in primary cultured dental pulp cells (DPCs), the results mimicking those observed in KN-3 cells treated with shRNA *Ift88*. Under this condition, the temporal pattern of DSPP expression took after that of IFT expression in DPCs. However, this result did not coincide with that of *Ift88* knockdown in KN-3 cells. In addition, inhibition of ciliogenesis was also observed through activation of canonical Wnt signaling pathway. Moreover, in DPCs, the accumulation of β -catenin gradually decreased with the progression of odontoblast differentiation. In contrast, the ratio of cilia-positive cells was increased.

These results collectively underscore the regulatory mechanism of canonical Wnt signaling pathway for the ALP activity through primary cilia, although it did not participate to DSPP expression level regulated through IFT88. Furthermore, our study also provides supportive evidence that canonical Wnt signaling pathway play an important role in ciliogenesis.

P1012

Board Number: B338

Single-molecule tracking of Smoothed reveals binding in the primary cilium that is altered by pathway agonists.

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Hedgehog (Hh) signaling plays an essential role in cell division and differentiation in embryonic and adult stem cells. Disruption of the pathway can often lead to fatal defects in embryos, as well as a wide variety of cancer types later in life. The pathway is initially switched on by the binding of the Hh ligand to the transmembrane protein Patched. Shortly after, another transmembrane protein, Smoothed (Smo), is derepressed and relocates to a small microtubule-structured organelle that protrudes from the cell's surface called the primary cilium. Once there, it influences members of the Gli family of transcription factors that then upregulate target genes in the nucleus. Although the ciliary structure is known to be very complex, standard fluorescence microscopy measurements have been hindered by the small dimensions (~400 nanometers in diameter and 2-5 microns long) which are on the order of the diffraction limit. To overcome this barrier, we have used highly sensitive, single-molecule microscopy to obtain the trajectories of individual Smo proteins on the surface of cilia with high temporal and spatial resolution (10 millisecond and 30 nanometers, respectively). By analyzing their movements, we have

observed three distinctive modes of motion: diffusion, directed motion, and binding, the last of which is altered in the presence of natural and synthetic small-molecule agonists.

P1013

Board Number: B339

An olfactory cilia pattern in the mammalian nose ensures high sensitivity to odors.

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By converting environmental signals into intracellular responses, cilia are critical for many developmental and physiological processes, including olfaction. Cilia in different cell types exhibit distinct morphologies with specific numbers and lengths, but little is known about what factors shape cilia morphology and how morphology impacts function. We aim to address these questions using the mouse olfactory system as a model. We recently discovered that olfactory cilia vary drastically in length depending on the cell location in the olfactory epithelium. Using specific markers to label subsets of olfactory sensory neurons (OSNs) in the whole-mount epithelia of young adult mice, we found that cilia length increases from ~2 μm in the posterior nasal septum to ~14 μm in the anterior septum, with the longest cilia (up to ~50 μm) typically found in the dorsal recess and ventral septum. Remarkably, the cilia length pattern is positively correlated with odorant absorption, as determined by a 3D computational fluid dynamics model of the mouse nasal cavity. We also found that cilia length modifies OSN function, since cells with longer cilia show higher sensitivity to odorant stimulation compared to their short cilia counterparts. Surprisingly, neither sensory experience nor neuronal activity are required for the formation and maintenance of the cilia pattern, but type III adenylyl cyclase (ACIII), a key olfactory signaling molecule and ubiquitous marker for primary cilia, dramatically alters cilia length. Together, these findings reveal a previously unrecognized configuration in the nose to ensure high sensitivity to odors and a critical role of ACIII in shaping sensory cilia, independent of odor-induced activity.

P1014**Board Number: B340****Probing the function of environmentally released Extracellular Vesicles in *C.elegans*.**J.S. Akella¹, J. Wang¹, L. Haas¹, M.M. Barr¹;¹Genetics, Rutgers University, Piscataway, NJ

Extracellular vesicles (ECVs) are membrane-bound organelles that mediate intercellular communication by aiding the exchange of a variety of cargo including receptors, nucleic acids, and peptides. ECVs are released by most cell types and are abundantly found in bodily fluids such as urine, breast milk, and semen. Immune cell-derived ECVs are known to play important roles in the regulation of immune response including antigen presentation and mediating the exchange of peptides and nucleic acids (Robbins P.D. et al; Nature Reviews Immunology 14,195–208(2014)). We recently found that a subset of the ciliated neurons of the nematode *C.elegans* release ECVs into the environment (Wang J. et al; Current Biology 24, 1–7(2014)). We are studying the in vivo functions of environmentally released ECVs.

Transcriptional profiling of the ECV releasing neurons of *C.elegans* showed a significant enrichment of genes involved in the innate immune response including PMK-1 (a p38 MAP kinase), TRF-1 (a Tumor Necrosis Factor Receptor Associated Factor), lectins and anti-microbial peptide(Wang J.et al; to be submitted). Enrichment of genes involved in the innate immune response in the ECV releasing neurons could be explained by their exposure to the environment and thus, to pathogens. PMK-1, a member of the stress activated p38 MAP kinase pathway plays an important role in the innate immune response of *C.elegans* to various pathogens by upregulating the expression of anti-microbial genes (Troemel E. et al; PLoS Genet 2(11): e183(2006)). Furthermore, we have found that mutants of *pmk-1* are defective in ECV release into their environment, suggesting a role for PMK-1 in ECV biogenesis and or release.

Human urinary ECVs are packed with anti-microbial factors and our preliminary data suggests that *C.elegans* ECVs are anti-microbial in nature. This finding lets us ask many questions that could provide insights into the roles of environmentally released ECVs. Are the anti-microbial factors induced by PMK-1 upon infection or stress packed into ECVs and released into the environment? Do ECVs isolated from mutants of *pmk-1* have different bioactivity in immunological assays? Are mutants that have defects in ECV biogenesis or environmental release more susceptible to infections? If yes, can this be rescued by applying wild type derived ECVs? Together, our *C.elegans* model will allow us to address fundamental questions regarding the bioactive functions of ECVs in vivo.

P1015**Board Number: B341****Dzip1 regulates the ciliary translocalization of smoothed for activation of the hedgehog pathway.**G. Wang¹, B. Zhang¹, Q. Jiang¹, C. Zhang²;¹School of Life Sciences, Peking University, Beijing, China, ²Peking Univ Coll Life Scis, Beijing, China

The hedgehog pathway plays important roles in regulating cell proliferation and individual development, and its hyper-activation often leads to tumorigenesis in humans. In most vertebrate cells, the primary cilia transduce the hedgehog signaling through providing a unique space for the localization of smoothed (Smo) and other components of this pathway. It is shown that Smo interacts with the BBSome, a vesicle-associated protein complex that provides sorting signals for cargo proteins destined to the cilia, via its C-terminal. However, how BBSome assembly is linked with the translocation of Smo into the cilia and consequently the activation of the hedgehog pathway is poorly understood. Here we report that Dzip1, a centrosome protein which regulates cilia assembly, is required for the activation of the hedgehog pathway via regulating the ciliary translocation of Smo. Furthermore, Dzip1-knockdown leads to sequestration of Smo in Rab8-positive vesicles in cytoplasm when the pathway is activated. Mechanically, Dzip1 interacts with the BBSome, and Dzip1-depletion leads to the absence of Smo from the BBSome. Together, our data revealed an important role of Dzip1 in governing the interaction of Smo with the BBSome and the activity of the hedgehog pathway.

Centrosome Assembly and Functions 2**P1016****Board Number: B343****Stress promotes non-random spindle pole body inheritance through the Pkc1 cell wall integrity pathway in budding yeast.**J. Lengefeld¹, Y.P. Barral²;¹Swiss Fed Inst Technol, Zürich, Switzerland, ²Institute of Biochemistry, ETH Zurich, Zurich, Switzerland

Age-dependent centrosome/spindle pole body (SPB) segregation is observed across many asymmetrically dividing eukaryotic cells. In budding yeast, SPBs are mainly conservatively duplicated i.e. an 'old' SPB is inherited from the previous mitosis and drives the assembly of the second newly synthesized 'young' SPB. Remarkably, the old SPB is segregated into the bud whereas the young SPB is kept within the mother cell. In this process, the SPB component Nud1, a protein related to mammalian centriolin, plays a central role by specifying SPB identity via the mitotic exit network (MEN; mammalian Hippo signaling pathway). However, the biological relevance and the mechanism of non-random SPB inheritance still remain unclear.

We studied the effect of environmental stress on age-dependent SPB inheritance. We observed that the stress induced by (long-term) fluorescence microscopy improves non-random SPB inheritance in MEN mutant cells. We provide evidence that the protein kinase C (Pkc1) cell wall integrity pathway is involved in this process: An overactive Pkc1 mutant under non-stress conditions improves age-dependent SPB and the disruption of the kinase Bck1 (downstream factor of Pkc1) abolishes the improvement of age-dependent SPB segregation under high stress conditions.

Together, our results indicate under stress conditions the Pkc1 cell wall integrity pathway promotes non-random SPB inheritance. While under non-stress conditions the previously described MEN pathway ensures faithful SPB inheritance, under stress conditions non-random SPB inheritance cannot be fully achieved via the MEN pathway, but is compensated by the Pkc1 signaling pathway. This indicates that the biological relevance of non-random SPB inheritance may be of particular importance under stressful conditions.

P1017

Board Number: B344

Chemical genetic analysis of Polo-like kinase 4 activity.

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Centrioles are small tube-like structures that form the core of the centrosome, the major microtubule-organizing center of the cell that plays a central role in most microtubule-related functions, including cell motility, formation of cilia, intracellular transport and chromosome segregation in mitosis. For centrioles to coordinate these diverse cellular processes their biogenesis and copy number must be strictly controlled. Centrioles normally duplicate in a tightly coordinated process that ensures each centriole reproduces only once per cell cycle. Polo-like kinase 4 (Plk4) has emerged as a dose-dependent, master regulator of centriole biogenesis: excessive levels of Plk4 result in the production of supernumerary centrioles while loss of kinase activity causes a failure of centriole duplication. Studying Plk4 kinase activity in vivo has proven to be a challenge due to the incredibly low cellular abundance of the protein and a lack of rapid and specific molecular tools to modulate Plk4 activity in vivo. Here, we describe a system by which we use CRISPR genetic engineering technology to knock-in an analog-sensitive (AS) mutation into both alleles of Plk4. This mutation does not affect the kinase's normal function, but does allow it to be specifically inhibited by the bulky ATP-analog 3-MB-PP1. We show that this cell line allows for rapid and reversible control of Plk4 activity. Thus, the Plk4 AS cell line is a novel tool to examine the immediate effects of Plk4 inhibition.

P1018**Board Number: B345****Molecular basis of ordered Plk4 binding to two distinct scaffolds critical for centriole biogenesis.**J. Park¹, S. Park¹, T. Kim¹, J. Kim², M. Kwak³, B. Ku², L. Tian⁴, S. Kim², B. Oh³, W. Yang⁴, K. Lee¹;¹NIH/NCI, Bethesda, MD, ²Korea Research Institute of Bioscience and Biotechnology, Daejeon, Korea,³Korea Advanced Institute of Science and Technology and Biotechnology, Daejeon, Korea,⁴NIH/NIDDK, Bethesda, MD

Polo-like kinase 4 (Plk4) is a key regulator of centriole duplication, an event critical for the maintenance of genomic integrity. How Plk4 is regulated at subcentrosomal structures remains unknown. Human Plk4 binds two distinct centrosomal scaffold proteins, Cep192 and Cep152, which form concentric ring structures around a centriole. We observed that Plk4 colocalizes with Cep192 around a daughter centriole in early G1, and then relocates to the outer Cep152 ring, which assembles around the Cep192-encircled daughter centriole in late G1 as Cep152 is recruited to this site. Plk4 cryptic polo-box domain (CPB) interacted with Cep192 (201–258) or Cep152 (1–60) fragment with a K_d of 177 ± 23 nM or 32 ± 4 nM, respectively. Analyses of the X-ray crystal structures of apo-CPB or CPB in complex with Cep192 (201–258) or Cep152 (1–60) revealed that, unlike the previously reported *Drosophila* CPB proposed to form a pseudosymmetric “four-winged” homodimer with both PB1-PB1 and PB2-PB2 contacts, the CPB of human Plk4 formed a symmetrical “X-shaped” homodimer with a PB2-PB2 interface. Cep192 (201–258) and Cep152 (1–60) bound to the same concave surface of CPB along the $\alpha 1$ of PB1 in opposite orientations and in a mutually exclusive manner. Consistent with their incompatible binding nature and differential CPB-binding affinities, *in vitro* biochemical experiments showed that Cep152 (1–60) effectively snatches the CPB away from a preformed CPB-Cep192 (201–258) complex, but not vice versa. Notably, a cancer-associated Cep152 (V8A) or (E21K) mutation impaired in the Plk4 CPB interaction induced defects in procentriole assembly and chromosome segregation. Thus, Plk4 is intricately regulated in time and space through ordered interactions with two distinct scaffolds, Cep192 and Cep152, and this unidirectional scaffold switching from Cep192 to Cep152 is likely critical to promote Plk4-mediated centriole biogenesis and to prevent aneuploidy that leads to cancers in humans.

P1019**Board Number: B346****Characterizing centriole biogenesis with auxin-inducible destruction of endogenous Plk4.**B.G. Lambrus¹, A.J. Holland¹, K.M. Clutario¹;¹Molecular Biology and Genetics, Johns Hopkins University School of Medicine, Baltimore, MD

Centrioles are small, cylindrical structures that nucleate centrosomes and play a key role in orchestrating various microtubule-related processes, such as cell motility, cilia formation, and mitotic spindle

organization. In order to ensure the proper coordination of these diverse functions, centriole biogenesis and copy number must be tightly controlled. In cycling cells, centrioles are restricted to duplicate precisely once per cycle. Though the mechanism of this duplication remains poorly understood, Polo-like kinase 4 (Plk4) is recognized as a dose-dependent, master regulator of centriole copy number. Previous efforts to study how Plk4 acts to coordinate centriole duplication have been hindered due to the technical difficulty of manipulating Plk4 protein levels with temporal precision. We have developed a tool to overcome this challenge. Application of the *auxin-inducible degradation (AID) system* in RPE-1 cells allows induction of rapid and specific depletion of Plk4, degrading 90% of endogenous Plk4 levels within an hour of induction. Chronic depletion of Plk4 leads to a p53-dependent growth arrest. However, disruption of p53 enables continued proliferation, and the development of acentriolar cells. By washing out auxin and thereby allowing the re-accumulation of Plk4, we observe *de novo* centriole formation. We will describe how we use this assay to study the dynamics and molecular basis of *de novo* centriole formation.

P1020

Board Number: B347

Centriole maturation requires regulated Plk1 activity during two consecutive cell cycles.

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Centrioles are conserved microtubule-based organelles that initiate the assembly of centrosomes and cilia. After initiation of their formation, young centrioles undergo a series of biochemical and structural modifications needed to acquire a competence for pericentriolar material organization, duplication, and cilia assembly. This ill-understood centriole maturation process is almost two cell cycles long and as a consequence, each cycling cell contains three generations of centrioles. Only one centriole per cell (the oldest one) is fully structurally mature and decorated with subdistal and distal appendages, which are needed for microtubule anchoring during interphase and for ciliogenesis, respectively. Appendages first form on the centriole by the end of its second mitosis, well after the centriole has already acquired its full length, organized a PCM, and undergone duplication in the previous cell cycle. Mechanisms that regulate appendage formation remain elusive. Previous work published by us and others indicated that Plk1 promotes centriole maturation. To better understand the effects of Plk1 on biochemical and structural centriole maturation we conducted a series of biochemical and correlative light and electron microscopy-based experiments. Our analysis revealed that controlled Plk1 activity during two consecutive cell cycles is required for gradual biochemical and structural maturation of the centrioles and timely appendage assembly. We found that inhibition of Plk1 impedes accumulation of appendage proteins and appendage formation on young centrioles. Exogenous expression of Plk1, either in cycling or interphase-arrested cells, stimulates premature accumulation of distal appendage proteins Cep164, Odf2/Cenexin, FBF1, SCLT1 and ccdc41, and assembly of appendages on younger centrioles, thus eliminating the age difference among the centrioles within one cell. These findings provide a new

understanding of how the centriole cycle is regulated and how proper cilia and centrosome number is maintained in the cells.

P1021

Board Number: B348

Loss of block-to-centriole reduplication without a loss of centriole orthogonal orientation in human cells.

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Centrioles duplicate by formation of a daughter centriole in orthogonal orientation to the wall of a mother centriole. This configuration is preserved throughout interphase until mother and daughter centrioles separate at the end of mitosis. The orthogonal configuration between the two centrioles serves as an intrinsic block-to-centriole reduplication because the mother centriole cannot further duplicate unless this configuration is lost. The molecular mechanisms leading to the loss of block-to-centriole reduplication are not currently known. It has been proposed that disorientation between a mother and a daughter centriole at the end of mitosis requires APC/C-dependent proteolytic events. To better understand the role of centriole configuration in maintaining a block-to-centriole reduplication in human cells, we analyzed structural changes occurring at the centrosomes undergoing reduplication by a combination of live-, fixed-cell light microscopy and electron microscopy. Our results demonstrate that centrioles can reduplicate in cycling and S-phase arrested human cells before a loss of orthogonal orientation is detectable by live cell recordings or electron microscopy and, before global proteolytic events occur at the centrosome. We propose that a loss of orthogonal orientation is a facultative event following a removal of block-to-centriole reduplication. This data is consistent with the data obtained in insect cells (Vidwans et al, 2002; Gottardo et al, 2014) revealing new details in the process of the formation of a human centriole.

P1022

Board Number: B349

Asterless licenses daughter centrioles to duplicate for the first time in *Drosophila*.

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Centrioles form centrosomes and cilia, and defects in any of these three organelles are associated with human disease. Centrioles duplicate once per cell cycle when a mother centriole assembles an adjacent daughter during S-phase. Daughter centrioles cannot support the assembly of another daughter until they mature into mothers during the next cell cycle [1-4], however the molecular nature of this daughter-to-mother transition has remained mysterious. Using quantitative live imaging in *Drosophila*

embryos we show that the conserved centriole duplication protein Asterless (*Drosophila* Cep152) is not incorporated into daughter centrioles as they assemble during S-phase, but is only incorporated once mother and daughter separate at the end of mitosis. The initial incorporation of Asterless (Asl) is irreversible, requires DSas-4, and is essential for daughter centrioles to mature into mothers that can support centriole duplication. We therefore propose a “dual licensing” model where Asl incorporation provides a permanent primary license to allow new centrioles to duplicate for the first time, while centriole disengagement provides a reduplication license to allow mother centrioles to duplicate again. Our preliminary data suggests that the primary licensing of new centrioles through the incorporation of Asl is regulated by cell cycle-dependent phosphorylation, which alters the DSas-4 -Asl interaction.

References: 1: Kleylein-Sohn et. al. (2007) *Dev Cell* (13) 190-202. 2: Cunha-Ferreira et al. (2009) *Curr Biol* (19) 43-49. 3: Loncarek et al. (2008) *Nat Cell Biol* (10) 322-328. 4: Wang et al. (2011) *JCB* (193) 727-739.

P1023

Board Number: B350

The mechanism of maternal centriole elimination in starfish oocytes.

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Centriole duplication and segregation is tightly coupled to the cell cycle in somatic cells to preserve centriole number over cell generations. However, at fertilization, upon gamete fusion, mechanisms have to exist to prevent the surplus of centrioles in the embryo that would lead to multipolar spindles and aneuploidy during early development. Therefore, centrioles are eliminated from the oocyte and only the sperm provides active centrioles to the zygote. Although this process is essential and common to animal species, the mechanisms underlying centriole elimination in female gametes are still poorly understood.

In most species, centrioles are eliminated during the long prophase of female meiosis that is difficult to access experimentally. In contrast, in starfish (*P. miniata*) oocytes, centriole elimination occurs during the rapid and synchronous meiotic divisions that can be easily followed by live-imaging. Therefore, to visualize centrioles *in vivo*, we established GFP markers and found that two pairs of centrioles are present at meiosis onset and contribute to spindle organization. Out of these two pairs, one is extruded into the first polar body at the end of the first meiotic division (MI). As no S-phase exists between MI and the second meiosis (MII), single centrioles form the poles of the MII spindle. Remarkably, by using the mother centriole specific marker Odf2-mEGFP, we found that the older, mother centriole always localizes to the spindle pole facing the plasma membrane. Consequently, the mother centriole is extruded into the second polar body, whereas a single daughter centriole remains in the mature egg. We additionally show, by tracking centrioles in live cells and by correlative electron microscopy, that the mother centriole is specifically transported to the plasma membrane at the end of MI, where it remains stably anchored. This likely occurs via the mother centriole specific appendages, in a mechanism that might be related to basal body formation. Additionally, the specific extrusion of mother centrioles is

functionally important, because if the centrioles are artificially retained in the egg (by inhibition of the polar body extrusion), only the mother centrioles remain active, recruit microtubules and cause multipolar spindles in the zygote.

Taken together, we propose the first comprehensive model for centriole elimination in animal oocytes that relies on the specific extrusion of the mother centrioles. We show that known and conserved features of the mother centrioles are employed in the oocyte for spindle positioning, membrane attachment and finally their own extrusion. Our observations suggest that the single daughter centriole remaining in the mature egg is not able to duplicate, leading to its elimination.

P1024

Board Number: B351

PLP forms novel centriole satellites and is critical for embryonic development.

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Centrosomes serve as the microtubule-organizing centers (MTOC) of most eukaryotic cells. Functional changes in centrosome behavior and activity are linked to oscillations in pericentriolar material (PCM) levels during the cell cycle. Elucidating how PCM dynamics are regulated is critical to understanding centrosome function throughout the cell cycle and how centrosome dysfunction drives pathologies, such as microcephaly and cancer. Pericentrin is a key regulator of the PCM. Intriguingly, we have identified a novel, cell cycle-dependent structural reorganization of Pericentrin-like-protein (PLP) in the early *Drosophila* embryo. We find PLP localizes to radially extended “satellites” specifically during interphase that partially colocalize with Centrosomin (Cnn) flares. Unlike flares, however, PLP satellites are not ejected from the centrosome, suggesting they may serve as structural components of the interphase centrosome. Clonal mutant analysis confirms that PLP satellites function primarily to organize the interphase PCM structure. Moreover, we find the Pericentrin/AKAP-450 centriole-targeting (PACT) domain is dispensable for PLP targeting to satellites or centrioles, but is required to maintain proper PLP satellite and PCM organization. Further, we demonstrate that PLP is critical for efficient microtubule (MT) organization, genome stability, and embryo viability. Collectively, our data argue that proper organization of the interphase PCM by PLP is essential for normal cell division and early development.

P1025**Board Number: B352****Chemically-induced reversible organelle knockout of centrioles reveals an essential role in the proliferation of non-transformed cells.**Y. Wong¹, J. Anzola², R. Davis², M. Yoon², A. Motamedi², A. Kroll¹, C. Seo², T. Gahman², A. Desai³, A. Shiau², K. Oegema³;¹Department of Cellular and Molecular Medicine, Ludwig Institute for Cancer Research, La Jolla, CA,²Small Molecule Discovery Program, Ludwig Institute for Cancer Research, La Jolla, CA, ³Ludwig Institute for Cancer Research, La Jolla, CA

Centrioles are microtubule-based organelles that direct the formation of centrosomes and cilia. Defects in centrosome structure and number are linked to aneuploidy and cancer, whereas ciliary dysfunction leads to a host of ciliopathies. Despite their broad impact on cell physiology, centriole function has been challenging to study in mammalian cells due to the lack of a facile methodology for specifically, persistently, and reversibly removing this organelle from cells. To facilitate analysis of centrioles, we developed centrinone – a specific small molecule inhibitor of PLK4, the kinase that initiates centriole assembly. Treatment with centrinone at sub-micromolar concentrations allows sustained depletion of centrioles from human and mouse cells, leading to loss of centrosomes and primary cilia. Depletion of centrioles from >30 human and mouse cell lines partitioned them into two distinct classes. Cell lines with a defective p53 pathway proliferated indefinitely in the absence of centrioles. G1+S and G2 durations were not altered, but spindle assembly was slowed and cells were more prone to mitotic defects that reduced population growth rate. Both centrioles and proliferation rate were restored upon centrinone washout. In cell lines with a wild-type p53 pathway, centriole removal triggered an irreversible p53-dependent G1 arrest within a few cell divisions. This arrest was not DNA damage signaling-dependent, and did not correlate with mitotic duration, indicating that the arrest triggered by centriole loss is distinct from the previously described prometaphase duration sensor. In transformed cell lines with different basal levels of centrosome amplification prior to centriole depletion, centrinone washout triggered a wave of de novo assembly and initial overduplication, followed by recovery to the level of amplification observed prior to centriole removal. Analysis of this recovery process indicated that overduplication is balanced by removal of cells with extra centrioles through multipolar mitosis and death. These results suggest that centriole number set points in cancer cell lines result from a dynamic equilibrium between centriole overduplication and removal of cells with extra centrioles, rather than being determined by historical overduplication events.

P1026**Board Number: B353****Cancer-wide survey of centrosomes reveals centriole fragmentation as a novel origin of supernumerary centrosomes.**G. Marteil¹, A. Guerrero², S. Godinho³, P. Machado^{1,4}, J. Loncarek⁵, S. Mendonça¹, I. Fonseca¹, D. Pellman⁶, M. Bettencourt-Dias¹;¹Instituto Gulbenkian de Ciência, Oeiras, Portugal, ²Instituto de Biotecnología Universidad Nacional Autónoma de México, Mexico, Mexico, ³Barts Cancer institute, London, United Kingdom, ⁴EMBL, Heidelberg, Germany, ⁵Laboratory of Protein Dynamics and Signaling, NIH/NCI-Frederick, Frederick, MD, ⁶Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA

A century ago, Theodor Boveri suggested that abnormalities in the major microtubule organising center of animal cells, the centrosome, lead to abnormal cell division and tumorigenesis. Those abnormalities have since been observed in cancer in vivo, but their incidence and causes remain poorly characterised. To address those important questions, we implemented a three-step screen and developed a state-of-the-art algorithm to score centriole number and length in 3D in the NCI-60 panel of cancer cell lines that represents cancer diversity. Importantly, we observed that the majority of these cell lines shows increase in both centriole number and length, and uncovered an important link between centrosome amplification and invasive capacity. Our work also suggests a novel origin of centriole amplification in cancer, which we subsequently validated with complementary approaches: as centriole length control is deregulated in cancer cells, centrioles can grow more than 3 fold and form an abnormal structure that fragments and generates smaller, functional centrioles. Centriole fragments then behave as microtubule organising centers leading to abnormal mitosis. The fact that centriole abnormalities are widespread in cancer, but not in normal cells, and the identification of novel causes of those abnormalities solidifies and suggests new avenues to the use of centrosomes in the clinic as diagnostic, prognostic and therapeutic tools.

P1027**Board Number: B354****A Novel Role for Primary Cilia in Centrosome Positioning and Division of Neural Progenitors.**T.J. Dantas¹, A. Baffet¹, A. Carabalona¹, R. Vallee¹;¹Department of Pathology and Cell Biology, Columbia University, New York, NY

Neural stem cells known as radial glial progenitors (RGPs) give rise to most of the neurons and glial cells in the developing mammalian cerebral cortex, as well as the stem cells of the adult. RGP cells are highly proliferative, and can divide symmetrically to increase the progenitor pool, or asymmetrically to produce neurons. The nucleus of the highly elongated RGP cell migrates basally during G1 and apically during G2, in a behavior known as interkinetic nuclear migration (INM). The RGP nucleus then enters mitosis and

divides exclusively at the apical terminus of the cell (Hu et al., 2013), located at the ventricular surface of the brain. The centrosome remains tethered at the apical terminus of the cell throughout the RGP cell cycle, and nucleates a primary cilium.

To determine the function of the primary cilium we first expressed ciliary markers in embryonic rat brain using in utero electroporation. We monitored primary cilia by high resolution light microscopic imaging of fixed and live brain tissue. In contrast to most cultured cell lines, we found primary cilia to project from the apical terminus of RGP cells throughout most of the cell cycle. To further test primary cilium function we interfered with ciliogenesis. Decreasing primary cilium assembly by disrupting IFT genes has been reported to alter hippocampal and cerebellar development via defects in sonic hedgehog signaling (Chizhikov et al., 2007; Han et al., 2008). However, we observe little effect of IFT inhibition on RGP cell behavior.

As a more definitive test of primary cilium function, we targeted earlier stages of ciliogenesis using RNAi or dominant negative cDNAs for centriolar distal appendage genes. In nonneuronal cells, this approach inhibits primary cilium assembly and also prevents the attachment of centrosomes to the plasma membrane (Tanos et al., 2013). In embryonic rat brain, disruption of some distal appendage genes resulted in a striking displacement of centrosomes from the apical terminus in a considerable fraction of RGP cells. This result suggests a completely novel role for ciliogenesis in restricting centrosome positioning to the apical terminus of RGP cells. A further consequence of interfering with distal appendage proteins was the appearance of ectopic mitotic events at substantial distances from the apical end of the cell at the ventricular surface.

These results strongly suggest that apical centrosome sequestration is triggered by the initial steps of ciliogenesis and maintained by the presence of the primary cilium throughout the cell cycle. In this manner, the primary cilium may be the responsible factor for restricting RGP mitosis to the ventricular surface of the brain and ensuring proper brain development.

P1028

Board Number: B355

Cyclin-dependent kinase control of centriole formation during differentiation of multiciliated epithelial cells.

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The centrosome is the microtubule-organizing center of animal cells, and at the core of each centrosome is a pair of centrioles. Centrioles are nine-fold symmetric microtubule structures that are required for formation of a cilium. The cilium is a critical signaling organelle and centriole and cilium dysfunction can lead to an array of diseases termed ciliopathies.

Along with chromosomal DNA, centrioles are the only structures that are duplicated once and only once per cell cycle. Centriole duplication and DNA replication are driven by the same cell cycle regulators, specifically the cell cycle kinase Cdk2 and its activators, cyclins E and A. In mammalian cells, Cdk2 in complex with cyclin E drives the G1-S transition, and in complex with cyclin A drives progression through S phase into G2-M. In a normal cell cycle centriole duplication and DNA replication are both initiated at the G1-S transition, and cannot occur again until after passage through mitosis. However, the two processes can be separated from each other in specialized cell types in which either centrioles or DNA are amplified. One example is multiciliated cells (MCCs) in which hundreds of centrioles form in the absence of DNA replication. We are interested in addressing the following question: how are two normally co-regulated cell cycle events separately regulated in differentiated cells?

MCCs exist in several locations of the mammalian body: the ventricles of the brain, fallopian tubes of the female reproductive tract, and respiratory tract. In cycling cells centrioles duplicate by forming adjacent to an existing centriole, whereas in MCCs most of the amplified centrioles form from granular structures termed deuterosomes. Molecular evidence indicates that despite the morphological differences, the same proteins and pathways are involved in centriole duplication and centriole amplification.

We show that Cyclin-dependent kinase2 (Cdk2), the major regulator of the S-phase events of DNA replication and centrosome duplication in cycling cells, is also required for centriole amplification without DNA replication in MCCs. We find that Cdk2 activity is required for centriole, but not deuterosome biogenesis. Interestingly, our results suggest that Cdk2 activity in these cells relies on the expression of a motile ciliogenesis-specific Cyclin activator.

P1029

Board Number: B356

Dissecting the role of PCNT in centriole engagement.

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Pericentrin (PCNT) is an integral component of the pericentriolar material (PCM) that serves as a scaffold for the anchoring of multiple proteins to the centrosome. Several human diseases are associated with PCNT mutations, one in particular is Majewski Osteodysplastic Primordial Dwarfism (MOPDII). The cause of MOPDII is biallelic loss-of-function mutations in PCNT, leading to loss of functional protein. To understand the molecular mechanism of MOPDII phenotypes, we engineered *Pcnt*^{-/-} mice and generated mouse embryonic fibroblasts (MEFs). Strikingly, *Pcnt*^{-/-} MEFs contained an increase in centrosome number compared to control, suggesting that *Pcnt* may be involved in centriole duplication. Concomitantly, we found that in *Pcnt*^{-/-} MEFs the centrioles lose 3 proteins out of 27 tested. Furthermore, these 3 proteins were immunoprecipitated with *Pcnt*. This subcomplex included two mother centriole appendage proteins, ninein and centriolin, and a protein previously reported to interact with *Pcnt*, Cep215. Centrosome duplication occurs once and only once per cell cycle. The first

step of centrosome duplication is the disengagement of the daughter centriole from the mother during the G1 phase of the cell cycle. If centrioles remain disengaged too long they will continuously form adjacent procentrioles, leading to centriole overduplication. The increase in overall centriole number in Pcnt^{-/-} MEFs suggests that Pcnt is involved in either centriole engagement or directly involved in duplication. We are currently investigating the molecular mechanism for Pcnt, and its newly identified interactions with centriolin, Cep215, and ninein, in the process of centriole engagement.

P1030

Board Number: B357

A unique set of centrosome proteins requires Pericentrin for spindle-pole localization and spindle orientation.

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Majewski Osteodysplastic Primordial Dwarfism type II (MOPDII) is caused by mutations in the centrosome gene pericentrin (PCNT) and leads to severe pre- and post-natal growth retardation. As in MOPDII patients, disruption of pericentrin (Pcnt) in mice caused a number of abnormalities including microcephaly, aberrant hemodynamics analyzed by in utero echocardiography, and cardiovascular defects, which were associated with mortality as in the human condition. To identify the mechanisms underlying these defects, we tested for deficiencies in cell and molecular function. All Pcnt^{-/-} mouse tissues and cells examined showed spindle misorientation. This mouse phenotype was associated with misdirected ventricular septal growth in the heart, decreased proliferative symmetric divisions in brain neural progenitors and increased misoriented divisions in fibroblasts; the same phenotype was seen in fibroblasts from three MOPDII individuals. Misoriented spindles were associated with disrupted astral microtubules and near complete loss of a unique set of centrosome proteins from spindle poles (ninein, Cep215, centriolin). All these proteins interact with Pcnt, suggesting that Pcnt serves as a molecular scaffold for this functionally-linked set of spindle pole proteins. Importantly, Pcnt disruption had no detectable effect on localization of proteins involved in the canonical cortical polarity pathway (NuMA,

p150glued, aPKC). Not only do these data reveal a spindle-pole-localized complex for spindle orientation, but they identify key spindle symmetry proteins involved in the pathogenesis of MOPDII.

P1031

Board Number: B358

Protein phosphatase 1 regulates ZYG-1 levels to limit centrosome duplication.

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In humans, perturbations of centrosome number are associated with tumorigenesis and microcephaly, therefore appropriate regulation of centrosome duplication is critical. Centrosome duplication involves building a single daughter centriole adjacent to each existing (mother) centriole and occurs once per cell cycle. The kinase Plk4 is essential for centriole biogenesis and its overexpression causes the formation of extra daughter centrioles in association with a single mother centriole. The *C. elegans* homolog of Plk4, ZYG-1, is required for centriole duplication, but our understanding of how ZYG-1 levels are regulated remains incomplete. We have identified the single PP1 β ortholog, GSP-1, and its regulators I-2^{SZY-2} and SDS-22 as controlling ZYG-1 levels. We find that down-regulation of PP1^{GSP-1} activity either directly, or by mutation of *szy-2* or *sds-22* can rescue the loss of centrosome duplication associated with the *zyg-1(it25)* mutation. This effect is a result of increased ZYG-1 levels, suggesting that PP1 normally antagonizes ZYG-1 proteins levels. Moreover we find that depleting PP1 activity in an otherwise wild-type embryo leads to centrosome amplification demonstrating the physiological relevance of PP1 action in modulating centrosome duplication.

P1032

Board Number: B359

SAS-6 Assembly Templated by the Lumen of Cartwheel-less Centrioles Precedes Centriole Duplication.

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Centrioles are 9-fold symmetric structures duplicating once per cell cycle. Duplication involves self-oligomerization of the centriolar protein SAS-6, but how the 9-fold symmetry is invariantly established remains unclear. Here, we found that SAS-6 assembly can be shaped by preexisting (or mother) centrioles. During S phase, SAS-6 molecules are first recruited to the proximal lumen of the mother centriole, adopting a cartwheel-like organization through interactions with the luminal wall, rather than via their self-oligomerization activity. The removal or release of luminal SAS-6 requires Plk4 and the cartwheel protein STIL. Abolishing either the recruitment or the removal of luminal SAS-6 hinders SAS-6

(or centriole) assembly at the outside wall of mother centrioles. After duplication, the lumen of engaged mother centrioles becomes inaccessible to SAS-6, correlating with a block for reduplication. These results lead to a proposed model that centrioles may duplicate via a template-based process to preserve their geometry and copy number.

P1033

Board Number: B360

Phosphorylation-driven Cnn scaffold assembly during centrosome maturation.

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Centrosomes comprise two major components: a pair of centrioles surrounded by pericentriolar material (PCM). The PCM expands dramatically in the process of centrosome maturation, functioning to nucleate and anchor microtubules (MTs). However the mechanism underlying this process remains unknown. Recently we have identified a domain (Phospho-Regulated-Multimerization PReM domain) in the *Drosophila* Centrosomin (Cnn) protein, which is phosphorylated by Polo/Plk1 at the centrosome during mitosis and is essential for PCM scaffold formation. Mutations that block the phosphorylation of PReM domain strongly disrupt the assembly of Cnn scaffolds, whereas phospho-mimicking mutations allow Cnn to multimerize in vitro and forming the spontaneous cytoplasmic scaffolds in vivo that organize MTs independent of centrosomes. Here, we present the further biochemical characterizations of this PReM domain to provide structural insights into the phosphorylation-driven self-interaction of Cnn molecules during centrosome maturation.

P1034

Board Number: B361

Stabilization of Cartwheel-less Centrioles for Duplication Requires CEP295-Mediated Centriole-to-Centrosome Conversion.

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Vertebrate centrioles lose their geometric scaffold, the cartwheel, during mitosis, concurrently with gaining the ability to recruit the pericentriolar material (PCM) and thereby function as the centrosome. Cartwheel removal has recently been implicated in centriole duplication, but whether "cartwheel-less" centrioles are intrinsically stable or must be maintained through other modifications remains unclear. Here, we identify a newborn centriole-enriched protein, KIAA1731/CEP295, specifically mediating

centriole-to-centrosome conversion but dispensable for cartwheel removal. In the absence of CEP295, centrioles form in the S/G2 phase and lose their associated cartwheel in mitosis but cannot be converted to centrosomes, uncoupling the two events. Strikingly, centrioles devoid of both the PCM and the cartwheel progressively lose centriolar components, whereas centrioles associating with either the cartwheel or PCM alone can exist stably. Thus, cartwheel removal can have grave repercussions to centriole stability, and centriole-to-centrosome conversion mediated by CEP295 must occur in parallel to maintain cartwheel-less centrioles for duplication.

Kinetochores Assembly and Functions 1

P1035

Board Number: B362

Tension-Based Elongation of *Drosophila* Kinetochores Protein CENP-C Promotes Stabilization of Kinetochores-Microtubule Attachments.

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Formation of stable kinetochores-microtubule (kt-MT) attachments is accompanied by movement of the binding interface away from the inner region of the kinetochores. In *Drosophila*, the outer kinetochores is ~40 nm further from the inner kinetochores at bioriented attachments than it is at unattached kinetochores. We refer to this 40 nm displacement between the inner and outer kinetochores as intrakinetochores stretch. It has been proposed that intrakinetochores stretch spatially repositions the attachment factors in the outer kinetochores beyond the reach of the Aurora B kinase activity centered at the inner centromere, thereby contributing to the establishment of stable bioriented attachments. We initially set out to identify the particular proteins that underpin intrakinetochores stretch in *Drosophila*. The protein CENP-C forms a link between the inner and outer regions of the kinetochores, binding to DNA and to centromere-specific nucleosomes through its C terminus and to the outer kinetochores through its N terminus. The intervening region of *Drosophila* CENP-C is predicted to lack secondary structure and contains no known, conserved structural motifs, and we hypothesized that this intrinsically disordered segment of the protein stretches under tension. After fluorescently labeling CENP-C at either end we determined, using super-resolution microscopy, that the protein lengthens by ~20 nm at bioriented attachments during metaphase. We next sought to examine the effect of artificially reducing the protein's length. To do so, we engineered a shortened, non-stretchable "minimal CENP-C" by removing 907 amino acids from the disordered region of the native protein. We verified that the shortened protein, unlike the full-length (1411 amino acid) version, does not elongate in the presence of tension at metaphase. Compared to wild-type cells, cells with the non-stretchable minimal CENP-C formed unstable kt-MT attachments and aligned fewer of their chromosomes at the metaphase plate. We also fused the C-terminal region of CENP-C directly to the Mis12 complex component Nsl1, producing a "hyper-shortened" version of the inner-to-outer kinetochores linkage. Cells with hyper-shortened CENP-C also formed unstable attachments and displayed an even greater

alignment defect than that seen in cells with minimal CENP-C. The alignment defect seen in both CENP-C mutants could be partially rescued by chemical inhibition of Aurora B activity. Cells with either shortened or hyper-shortened kinetochores also exhibited chaotic chromosome movements within the spindle that are suggestive of an inability to form stable kt-MT attachments. Efforts are now under way to rescue the deleterious effects associated with non-stretchable kinetochores by replacing the deleted region of CENP-C with peptide linkers of various lengths and physical properties, including other intrinsically disordered protein domains. Our results are consistent with a model in which tension-derived CENP-C elongation enables dephosphorylated outer kinetochore attachment factors to form stable kt-MT attachments.

P1036

Board Number: B363

CENP-C stabilizes CENP-A nucleosomes at the centromere.

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Centromere identity through cell cycles is maintained epigenetically by the presence of nucleosomes containing the histone H3 variant CENP-A. Consistent with a role as a stable epigenetic mark, we find that CENP-A nucleosomes are quantitatively retained at the centromere at which they are first assembled. The CENP-A nucleosome binding protein CENP-C is constitutively and directly bound to CENP-A nucleosomes at the centromere and is a prime candidate to stabilize CENP-A nucleosomes at the centromere. Indeed, using reconstituted centromere complexes, we find that CENP-C reshapes the histone core of the nucleosome, and rigidifies both surface and internal nucleosome structure. We find that depletion of CENP-C leads to the rapid removal of CENP-A from centromeres, indicating that CENP-C is necessary to retain CENP-A nucleosomes at the centromere. These data shed light on the molecular requirements of maintaining centromere identity, and lead to a model wherein CENP-A and CENP-C collaborate to maintain centromere identity.

P1037**Board Number: B364****Lateral to End-on Conversion of Chromosome-Microtubule Attachment Requires Kinesins CENP-E and MCAK.**R.L. Shrestha¹, V.M. Draviam¹;¹Department of Genetics, Univ Cambridge, Cambridge, England

Background: Proper attachment of chromosomes to microtubules is crucial for the accurate segregation of chromosomes. Human chromosomes attach initially to lateral walls of microtubules. Subsequently, attachments to lateral walls disappear and attachments to microtubule ends (end-on attachments) predominate. While it is known in yeasts that lateral to end-on conversion of attachments occurs through a multistep process, equivalent conversion steps in humans remain unknown.

Results: By developing a high-resolution imaging assay to visualize intermediary steps of the lateral to end-on conversion process, we show that the mechanisms that bring a laterally bound chromosome and its microtubule end closer to each other are indispensable for proper end-on attachment because laterally attached chromosomes seldom detach. We show that end-on conversion requires (1) the plus-end-directed motor CENP-E to tether the lateral kinetochore onto microtubule walls and (2) the microtubule depolymerizer MCAK to release laterally attached microtubules after a partial end-on attachment is formed.

Conclusions: By uncovering a CENP-E mediated wall-tethering event and a MCAK-mediated wall-removing event, we establish that human chromosome-microtubule attachment is achieved through a set of deterministic sequential events rather than stochastic direct capture of microtubule ends.

P1038**Board Number: B365****Centromere Protein-F Includes Two Sites that are Efficient Couplers to Depolymerizing Tubulin.**J. McIntosh¹, V. Volkov², W. Old¹, P. Grissom¹, V. Arzhanik², T. McClure-Begley¹;¹Univ Colorado, Boulder, CO, ²Ctr Theoretical Problems of Physical and Chemical Pharmacology, Moscow, Russia

The attachment of kinetochores to dynamic microtubules (MTs) is of central importance to accurate chromosome segregation at mitosis. Centromere Protein F (CENP-F) has been shown to include two MT binding domains, so it may participate in this key mitotic process. Here we show that a 558 amino acid fragment from the N-terminus of CENP-F forms dimers in solution that prefer binding to curled oligomers of tubulin relative to a MT wall by ~15-fold, suggesting that CENP-F may play a role in the firm bond that forms during prometaphase between a kinetochore and the plus ends of dynamic kinetochore MTs. This N-terminal fragment contains three coiled coils but studies using electron microscopy, cross-

linking, and mass spectrometry indicate that it folds to form a binding site that associates with β -tubulin at K392, a residue that lies near the inter-dimer boundary in a MT and close to the interface between adjacent protofilaments. A 522 amino acid fragment from CENP-F's C-terminus prefers taxol-stabilized MTs, but either of these CENP-F fragments can bind to and diffuse on the wall of a stable MT. Measurements of fluorescence brightness suggest that these fragments oligomerize when bound to a MT, and when they associate with dynamic tubulin polymers, they will follow the end of a shortening MT. When either of these binding domains is coupled to a microbead, the force it can transduce from a shortening MT averages ~ 5 pN but can exceed 13 pN, making CENP-F the most effective coupler to shortening MTs so far discovered outside the fungi.

P1039

Board Number: B366

Dissecting the Spatiotemporal Regulation of CENP-A Assembly.

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The proper segregation of chromosomes in mitosis requires the correct assembly of the kinetochore upon centromeric chromatin. Nucleosomes containing the centromere specific histone H3 variant, CENtromere Protein A (CENP-A), form the specialized chromatin of the centromere that specifically recruits proteins for centromere and kinetochore assembly. A central question in understanding centromere function is how new CENP-A is recruited and assembled to maintain centromeres through replication and division. This process must be carried out with spatial and temporal precision to protect against the formation of ectopic centromeres or the loss of centromeres, both of which can lead to chromosome missegregation and aneuploidy.

CENP-A assembly requires two protein complexes, the Mis18 complex and the CENP-A chaperone HJURP. These complexes are targeted to vertebrate centromeres in anaphase and G1 when new CENP-A assembly occurs. Mis18 complex recruitment is mediated in part through the direct binding of the Mis18 complex subunit M18BP1 to the conserved centromere protein CENP-C. Using an *in vitro* CENP-A assembly system in *Xenopus* frog egg extracts we showed that the interaction between CENP-C and M18BP1 occurs only in metaphase and that depletion of either CENP-C or M18BP1 inhibited new CENP-A assembly. Thus, the mitosis-specific interaction of CENP-C and M18BP1 may provide a mechanism to control the spatiotemporal regulation of CENP-A assembly.

Here we report the interaction domains for M18BP1 and CENP-C and demonstrate that the conserved SANTA domain of M18BP1 is required for its localization in both mitosis and interphase. In addition, we found that mutation of the SANTA domain disrupts the interaction between CENP-C and M18BP1 during mitosis, suggesting that regulation of this interaction is a key regulatory step in CENP-A assembly. We found that both the mitotic localization of M18BP1 and the interaction between CENP-C and M18BP1 are regulated by phosphorylation. To test whether the phosphoregulation of the interaction between CENP-C and M18BP1 is responsible for the cell cycle dependent localization of M18BP1 to centromeres,

we identified phosphorylation sites on M18BP1 in the interaction domain that binds CENP-C. We find that M18BP1 localization to centromeres and M18BP1 binding to CENP-C are controlled by different sets of phosphorylation sites. Taken together, our data supports a model where two distinct pathways control the localization of M18BP1 to metaphase centromeres, one through direct interaction with CENP-C and another through an as yet unidentified phosphorylation dependent mechanism.

P1040

Board Number: B367

Determinants of CENP-A for early steps in centromere establishment.

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The centromere is the chromosomal locus required for the accurate segregation of chromosomes during cell division. In many eukaryotes, including vertebrates, the centromere is marked epigenetically by the histone H3 variant Centromere Protein A (CENP-A). CENP-A assembles into nucleosomes at the centromere and forms the chromatin that recruits centromere components at the foundation of mitotic kinetochores. To dissect the roles of CENP-A at the centromere, gain-of-function histone H3 chimeras containing various regions, unique to CENP-A, have been particularly helpful. Instead of examining the requirements at established centromeres, here we investigated the earliest events we could measure in centromere establishment. To do this, we targeted human CENP-A or histone H3 chimeras to Lac operator (LacO) sequences integrated into a site on a chromosome arm by fusing each of the chimeras to the Lac repressor (LacI). Using this approach, we found surprising CENP-A requirements for initial recruitment of two essential constitutive centromere proteins (CENP-C and CENP-T) that differ from the requirements observed in genetic replacement experiments using the same H3 chimeras (Fachinetti et al., 2013, Nat Cell Biol. 9:1056-66). In particular, we found that the C-terminal tail of CENP-A is required but not sufficient for recruiting any detectable CENP-C to LacO. We also found that the recruitment of CENP-T requires three distinct regions of CENP-A: 1) a short region within the N-terminal tail of CENP-A, 2) the CENP-A targeting domain (CATD), and 3) the C-terminal tail of CENP-A. Taken together, our results indicate that the regions of CENP-A required for new centromere formation differ from those required for centromere propagation. In this way, our centromere establishment experiments help to reveal the regions of CENP-A involved in recruiting centromere components required for centromere function at mitosis that could otherwise be obscured in traditional genetic approaches by the complex intermolecular interaction network of centromere proteins.

P1041**Board Number: B368****Identification of Cdk-dependent phosphorylation sites on the CENP-A chromatin assembly complex by quantitative phosphoproteomics.**L.Y. Guo¹, X. Cao¹, A. Stankovic², L.E. Jansen², B.A. Garcia¹, B.E. Black¹;¹Department of Biochemistry and Biophysics, University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA, ²Instituto Gulbenkian de Ciência, Oieras, Portugal

Maintenance of genomic stability requires accurate propagation of centromere location with every cell division. Replication of centromeric DNA in S phase must be followed by loading of new CENP-A molecules into centromeric chromatin, to prevent loss of CENP-A nucleosomes from successive dilutions. But unlike canonical histones, CENP-A loading is not coupled to DNA replication, but rather occurs exclusively after mitotic exit. This unique timing is controlled by cyclin-dependent kinases 1 and 2 (Cdk1/2), whose activity prevents CENP-A loading until the decline of Cdk1/2 activity in telophase/G1, but the targets of Cdk regulation are unknown. By combining the powerful methods of stable isotope labeling by amino acids in cell culture (SILAC), affinity-purification of the CENP-A-containing chromatin assembly complex with HJURP and its partner histone H4, titanium dioxide enrichment of phosphopeptides, as well as high-resolution mass spectrometry, we quantitatively measured the changes of multiple phosphorylation sites on CENP-A-containing complexes upon Cdk inhibition. We will present the results of these experiments, including findings that putative Cdk sites on HJURP are heavily phosphorylated in early mitosis, and are rapidly dephosphorylated upon Cdk inhibition. In contrast, putative Cdk sites on the CENP-A N-terminal tail remain phosphorylated even after Cdk inhibition. All of these post-translational modifications are strong candidates to participate in the regulation of the CENP-A chromatin assembly pathway.

P1042**Board Number: B369****Localization and function of budding yeast CENP-A depends upon kinetochore protein interactions and is independent of canonical centromere DNA sequence.**K. Ho¹, D. Tsuchiya¹, A. Oliger¹, S. Lacefield¹;¹Indiana University Bloomington, Bloomington, IN

The assembly of kinetochore at the centromere is critical for accurate chromosome segregation in mitosis. Kinetochore assembly is initiated from the centromeric chromatin, which recruits the DNA-binding inner kinetochore and the microtubule-binding outer kinetochore subcomplexes to connect the chromosome to spindle microtubules. The kinetochore is structurally conserved in eukaryotic model organisms, but the underlying centromeric sequence is highly diverse without consensus sequence. Therefore, it has been proposed that the centromere is specified by epigenetic markers, such as the centromeric histone H3 variants, CENP-A. The only exception is in budding yeast, which has a small ~125

base pair point centromere that consists of three DNA sequence elements. Although the yeast centromeric DNA sequence is sufficient for de novo kinetochore assembly, tethering specific kinetochore proteins to a non-centromeric locus can induce the assembly of a functional synthetic kinetochore, suggesting that the centromeric DNA sequence is not necessary for kinetochore assembly. It has been shown that mutations of Cse4 (the yeast CENP-A) or centromeric DNA sequence abolish the localization of most kinetochore proteins at the centromere and lead to chromosome missegregation. However, it was not clear whether the DNA sequence and Cse4 only provide information to specify the locus for kinetochore assembly, or whether they are also involved in regulating kinetochore function. We used a synthetic kinetochore to address this question because it is assembled at an ectopic locus without the centromeric sequence. In combination with biochemical and genetic approaches, we found that Cse4 is localized to the ectopic kinetochore assembly site without centromeric sequence, and is required for the function of synthetic kinetochore. These results suggest that even in the point centromere in yeast, DNA sequence is not essential for Cse4 localization and the assembly of the kinetochore. Furthermore, our results indicate that Cse4 is part of a minimal functional kinetochore, and it is directly involved in regulating kinetochore function.

P1043

Board Number: B370

TRAMM is a moonlighting protein that functions in chromosome congression, kinetochore stability and CENP-E recruitment.

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Chromosome congression requires the stable attachment of microtubules to chromosomes mediated by the kinetochore, a large proteinaceous structure whose full complement of proteins is unknown. Here we present the unexpected finding that a protein we call TRAMM, that interacts with the TRAPP vesicle tethering complex, also plays a role in mitosis. Depletion of TRAMM results in non-congressed chromosomes and leads to arrest of cells in mitosis. TRAMM associates with chromosomes and its depletion affects a number of kinetochore proteins, the strongest effect being seen for CENP-E whose recruitment to the kinetochore is greatly reduced. TRAMM is phosphorylated early in mitosis and dephosphorylated during anaphase. Interestingly, this phosphorylation/dephosphorylation cycle correlates with its association/disassociation with CENP-E. Finally, we demonstrate that a phosphomimetic form of TRAMM recruits CENP-E to kinetochores more efficiently than does the non-phosphorylatable mutant. Our study identifies a moonlighting function for TRAMM during mitosis and adds a new component to kinetochores that regulates their stability. The migration from one large complex (TRAPP) during interphase to another large complex (kinetochore) during mitosis makes TRAMM unique amongst other moonlighting proteins.

P1044**Board Number: B371****A non-epigenetic component for maintenance of human centromere function dependent on the centromeric DNA binding protein CENP-B.**D. Fachinetti¹, J. Han², P. Ly², M. McMahon³, D.W. Cleveland⁴;¹Univ California-San Diego-LICR, La Jolla, CA, ²Ludwig Institute for Cancer Research, La Jolla, CA,³University of California, San Diego, San Diego, CA, ⁴Ludwig Institute for Cancer Research and Department of Cellular and Molecular Medicine, University of California, San Diego, CA

Human centromeres are specified by an epigenetic mark that identifies and maintains centromere function through a two-step mechanism relying on self-templating centromeric chromatin assembled with the histone H3 variant CENP-A, followed by CENP-A-dependent nucleation of kinetochore assembly. Nevertheless, natural human centromeres are positioned within specific megabase chromosomal regions containing α -satellite DNA repeats, a subset of which contains DNA sequence-specific binding sites for the centromere protein CENP-B. We now demonstrate the existence of a parallel, DNA sequence-dependent pathway for kinetochore assembly that is dependent on CENP-B. CENP-B is shown to bind both CENP-A's amino tail and CENP-C. These interactions are shown to reinforce epigenetically defined centromere function through maintaining CENP-C levels at centromeres and promoting CENP-A deposition if CENP-C levels are reduced. These data identify a non-epigenetic component for human centromere function mediated by DNA sequence-specific centromere binding of CENP-B.

P1045**Board Number: B372****Artificial dimerization of INCENP/Sli15 makes the essential biorientation function of Aurora B/Ipl1 independent of its localization to microtubules and to the inner centromere.**C. Campbell¹, A. Desai², K. Turnbull²;¹University of California, San Diego, La Jolla, CA, ²Ludwig Institute for Cancer Research, La Jolla, CA

Accurate segregation of the replicated genome requires biorientation of chromosomes on the metaphase spindle, which is ensured by the kinase Aurora B/Ipl1, a member of the 4-subunit chromosomal passenger complex (CPC). In prometaphase, Aurora B localizes between sister kinetochores at the inner centromere and this localization is dependent on the CPC subunit Survivin. We had previously shown in budding yeast that disrupting inner centromere CPC localization, by truncating the N-terminus of the INCENP/Sli15 scaffold of the CPC to eliminate association with Survivin/Bir1, did not affect chromosome segregation during mitosis and meiosis (Campbell & Desai. Nature 2013 497:118-21). As N-terminally truncated Sli15, in contrast to wildtype Sli15, concentrated prematurely on pre-anaphase spindles, we proposed that the microtubule-binding activity of Sli15 was necessary for

bioorientation in the absence of inner centromere localization. Consistent with this idea, we show here that further truncation of Sli15 into the central, microtubule-binding region is lethal. Expression of a fragment (Sli15CT) that contains only the C-terminal 199 amino acids of the 698 aa-long Sli15 results in chromosome missegregation equivalent to a null mutant. Surprisingly, Glutathione-S-Transferase (GST)-mediated dimerization of Sli15CT rescued the lethality observed with Sli15CT and resulted in only mild chromosome segregation defects. In addition, GST-Sli15CT no longer localized prematurely to pre-anaphase spindles, indicating that dimerization compensates for microtubule localization. Cumulatively, these results indicate that the CPC can sense tension and correct misattachments independently of its concentration on either chromatin or microtubules. These results are consistent with a model in which physical clustering activates Aurora B/Ipl1 and kinetochore-intrinsic sensing of tension is sufficient for chromosome bioorientation.

P1046

Board Number: B373

CENP-E kinesin exhibits enhanced motility on detyrosinated microtubules.

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Kinetochore-localized kinesin-7, CENP-E, is a microtubule plus end-directed motor, which is responsible for the transport of pole-proximal chromosomes from the spindle poles to the spindle equator. The plus ends of astral microtubules nucleated from the spindle poles point toward the cell cortex. However, the pole-proximal chromosomes must be transported to the spindle equator, while avoiding moving along astral microtubules towards the cell cortex. Interestingly, spindle microtubules pointing to the equator, but not the astral microtubules, are enriched in alpha-tubulin lacking the C-terminal tyrosine residue, so-called detyrosinated tubulin. To test if microtubule detyrosination affects CENP-E motility, we performed a series of in vitro assays with purified CENP-E kinesin and fully detyrosinated or tyrosinated human tubulin. Using single molecule TIRF microscopy with microtubules polymerized from these two different tubulins, we show that CENP-E walks faster and for longer distances on detyrosinated microtubules. Our preliminary force measurements using an optical trap system suggest that the CENP-E kinesin can sustain higher forces on detyrosinated vs. tyrosinated microtubules, implying that microtubule detyrosination is an important regulatory event in the specification of microtubules within the mitotic spindle. We propose that the enhanced motility of CENP-E motor on detyrosinated microtubules is responsible for the preferential motion of polar chromosomes toward the spindle equator.

P1047**Board Number: B374****CENP-E and detyrosinated microtubules guide peripheral polar chromosomes towards the cell equator.**M. Barisic¹, P. Aguiar², S. Geley³, H. Maiato^{1,4};¹Chromosome Instability Dynamics Laboratory, Instituto de Biologia Molecular e Celular, Universidade do Porto, Porto, Portugal, ²INEB, Universidade do Porto, Porto, Portugal, ³Biocenter, Innsbruck Medical University, Innsbruck, Austria, ⁴Cell Division Unit, Department of Experimental Biology, Faculdade de Medicina, Universidade do Porto, Porto, Portugal

Accurate chromosome segregation during cell division in metazoans relies on proper chromosome congression at the equator. Chromosome congression is supported by the coordinated action of motor proteins that slide misaligned chromosomes along pre-existing spindle microtubules. These proteins include the minus end-directed kinetochore motor Dynein, and the plus end-directed motors CENP-E at kinetochores and chromokinesins on chromosome arms. However, how these opposite and spatially distinct activities are coordinated to drive chromosome congression remains unknown. Here we used RNAi, chemical inhibitions, kinetochore tracking and laser microsurgery to uncover the functional hierarchy between kinetochore and arm-associated motors, exclusively required for congression of peripheral polar chromosomes in human cells. We show that Dynein poleward force counteracts chromokinesins to prevent stabilization of immature/incorrect end-on kinetochore-microtubule attachments and random ejection of polar chromosomes. At the poles, CENP-E becomes dominant over Dynein and chromokinesins to bias chromosome ejection towards the equator. Finally, we show that experimental inhibition of tubulin detyrosination in living human cells dissociates CENP-E from microtubules and impairs congression of peripheral polar chromosomes, similarly to CENP-E depletion/inhibition. This bias for detyrosinated microtubules explains the dominant and selective role of CENP-E in sliding polar chromosomes exclusively towards the equator.

P1048**Board Number: B375****Kinetochore architecture encodes a mechanical toggle-switch to control the spindle assembly checkpoint.**P. Aravamudhan¹, A.A. Goldfarb², A.P. Joglekar^{1,2};¹Biophysics, University of Michigan, Ann Arbor, MI, ²Cell and Developmental Biology, University of Michigan, Ann Arbor, MI

How microtubule attachment to the kinetochore silences the Spindle Assembly Checkpoint (SAC) has puzzled cell biologists for over two decades. It is clear that the architecture of kinetochore changes following microtubule attachment, and that this altered architecture disrupts SAC signaling. However,

the network of protein complexes that forms the kinetochore can change in innumerable ways following microtubule attachment, and one or more of these changes can disrupt any of the steps in SAC activation. Therefore, defining the molecular mechanism that enables this attachment sensitive signaling has been challenging. To overcome this challenge, we asked how the well-defined architecture of attached kinetochore disrupts SAC signaling. We show that microtubule attachment to the kinetochore changes the separation between two conserved kinetochore proteins, Ndc80 and Spc105 to disrupt phosphorylation of Spc105, an essential step in SAC activation.

We used a comprehensive analysis of the activity of SAC proteins artificially localized at specific kinetochore positions to find that the architecture of attached kinetochore prevents Mps1 kinase from phosphorylating Spc105. Furthermore, attachment could control the phosphorylation of Spc105 only when Mps1 was localized in the outer kinetochore, proximal to the microtubule-binding domain of Ndc80. Mps1 localized in the inner kinetochore constitutively activated the SAC, irrespective of the attachment state. This suggested that Mps1 must bind to the outer kinetochore to enable attachment-dependent SAC signaling. An engineered sensor for Mps1 activity revealed that this was the case. These observations, when combined with structural and biochemical properties of Ndc80 and Spc105 suggested a simple toggle-switch like mechanism that controls the SAC: In unattached kinetochores, the microtubule-binding domain of Ndc80 and the phosphodomain of Spc105 are in close proximity. This allows Mps1 bound to Ndc80 to phosphorylate Spc105 and turn the SAC on. Microtubule attachment to the kinetochore separates the two domains, and prevents phosphorylation of Spc105 to turn the SAC off. FRET between the microtubule-binding domain of Ndc80 and the phosphodomain of Spc105 confirmed that the two domains are in close proximity (less than 10 nm) in unattached kinetochores, and are separated by microtubule attachment. We further demonstrated the working of the toggle-switch by artificially reducing the separation between its terminals: moving the phosphodomain of Spc105 close to the microtubule binding domain of Ndc80 activated the SAC irrespective of the attachment state of the kinetochore. Our results demonstrate how a specific change in the architecture of the kinetochore disrupts SAC signaling.

P1049

Board Number: B376

Reconstitution of strong kinetochore attachments requires more than the microtubule binding components.

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The binding strength of a native kinetochore is greater than that provided by the sum of its components. The kinetochore must retain a grip on the ends of dynamic microtubules as they proceed through phases of assembly and disassembly while under tension. If the grip is too loose, kinetochores release; if

the grip is too tight, they detach with the disassembling tubulin subunits. Native kinetochore particles with no more than 20 copies of each mechanical arm bind to dynamic microtubules with a lifetime of 40 minutes at physiologically relevant forces. The Ndc80 complex (Ndc80c) is a key microtubule-binding component of the kinetochore that is essential for viability, but only weakly binds microtubules and cannot alone recapitulate the behavior of a kinetochore. Even ~100 copies of Ndc80c on a bead achieves an attachment lifetime of only 40 seconds with an average rupture force of 2.5 pN. Previous studies have shown that combining Ndc80c with another outer kinetochore complex, Dam1c, enhances its microtubule affinity, its ability to track with dynamic tips, and its capacity to remain tip-bound under load. Dam1c and Ndc80c together yield a rupture force of 7.5 pN, significantly weaker than purified kinetochore particles, which rupture at 9 pN on average. We found that the central kinetochore complex MIND, which does not directly bind microtubules, can also enhance the strength and affinity of Ndc80c for microtubules. Using total internal reflection fluorescence (TIRF) and optical trap microscopy we demonstrate that addition of MINDc increases the residence time of Ndc80c on microtubules by 6-fold and the strength of microtubule attachments nearly 2-fold. MINDc and Ndc80c bind with a 1:1 stoichiometry; therefore the mechanism of activation cannot be simply due to oligomerization of Ndc80c by MINDc. Intriguingly, the MIND complex binds Ndc80c far away from its microtubule-binding domain, suggesting that it allosterically enhances Ndc80c-microtubule interactions. Combining the Dam1 and MIND complexes with Ndc80c additively enhances its microtubule binding and strengthens the microtubule attachment to 9 pN, the level of purified native kinetochores. This correspondence suggests that the load-bearing capacity of the kinetochore can be recapitulated using only 3 of the 8 kinetochore complexes, highlighting the importance of these complexes in maintaining strong attachments. These results highlight the individual and combined contributions of kinetochore complexes at the molecular level, providing a better understanding of kinetochore function and regulation during cell division.

P1050

Board Number: B377

Manipulating mitotic kinases through chemical dimerization: increased Aurora B or Mps1 activity at metaphase kinetochores drives chromosomes out of alignment.

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The mitotic kinases Aurora B and Mps1 play critical, inter-related roles at kinetochores and are required for both chromosome alignment and mitotic checkpoint activity. Much of our knowledge of the functions of these kinases is built on loss-of-function experiments. As a complementary approach, we sought to test the effects of increasing their activity at kinetochores during metaphase, when the activity of both kinases is normally minimal. We used chemically-induced dimerization to artificially recruit either Aurora B or Mps1 to metaphase kinetochores with temporal precision. In both cases the

metaphase plate became progressively disordered as chromosomes migrated towards spindle poles. Both perturbations destabilized kinetochore-microtubule attachments, caused Mad2 to accumulate at kinetochores and prevented cells from entering anaphase. The disruptive effect of hyperactivating Aurora B at kinetochores is consistent with prevailing models of Aurora B function and with earlier results using constitutively kinetochore-tethered Aurora B. By contrast, the effects of Mps1 activity on kinetochore-microtubule interactions were unexpected. These results motivate future experiments investigate whether Mps1 acts through direct destabilization of kinetochore-microtubule interactions, by proxy through Aurora B activation, or some combination of both processes.

P1051

Board Number: B378

Molecular Mechanisms Regulating Localization and Proteolysis of Centromeric Histone H3 Variant Cse4.

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Cse4p is an essential centromeric histone H3 variant in *Saccharomyces cerevisiae* that defines centromere identity. Overexpression and mislocalization of CENP-A (human homolog of Cse4) has been observed in several colorectal cell lines (Tomonaga et al. 2003). Budding yeast is an ideal model system to identify genes/pathways that can be targeted for selective lethality in cancer cells that show overexpression and mis-localization of CENP-A. Mis-localization of Cse4 is observed in yeast *spt4Δ*, *cac1Δhir1Δ*, *snf2Δ* and *psh1Δ* mutants. Proteolysis of Cse4/Cid prevents mis-localization of these proteins in yeast and flies. Psh1, an E3 ligase, interacts with the C-terminus of Cse4 and regulates its ubiquitin-mediated proteolysis. Despite ubiquitination of Cse4 by Psh1, Cse4 is only partially stabilized in *psh1Δ* strains, and the interaction of Psh1 with the C-terminus of Cse4 is not sufficient for proteolysis suggesting that additional factors and domains within Cse4 regulate its proteolysis. Studies from our laboratory are aimed at: a) defining the region(s) of Cse4 that mediate its proteolysis to prevent mis-localization to non-centromeric regions and b) characterizing pathways for proteolysis of Cse4 for faithful chromosome segregation. We provided evidence for a direct relationship between mislocalization of Cse4, and chromosome loss in budding yeast (Au et al., 2008) and showed that the N-terminus of Cse4p regulates its ubiquitin-mediated proteolysis for faithful chromosome segregation (Au et al., 2013). We performed genome-wide synthetic lethal screens with GALCSE4 to identify and characterize pathways that regulate the cellular levels of Cse4. The results of the screen has yielded a 'gold mine' of data and provided a global genetic view into potential mechanisms to target cancers overexpressing CENP-A. Our results represent the first identification for essential genes that encode components of the evolutionarily conserved ubiquitin ligase (SCF) and replication dependent kinases (DDK) complexes in Cse4 proteolysis. For non-essential genes, we identified PSH1 as well as multiple components of multi-subunit complexes such as the replication-independent histone chaperone complex (HIR1, HIR2, HIR3 and HPC2) within the top ten most significant hits of the screen. We have used multiple approaches to validate the roles of SCF, DDK complexes and histone chaperones in Cse4

proteolysis. We will present a working model showing how multiple pathways regulate the ubiquitin-mediated proteolysis of Cse4 and localizatoin. Given the evolutionary conservation of the yeast genes we will pursue studies with the human homologs to identify pathways that when disrupted lead to selective killing of cancer cells overexpressing CENP-A.

P1052

Board Number: B379

Plk1-dependent timely delocalization of the PBIP1-CENP-Q complex from kinetochores is critical for proper chromosome segregation.

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Mammalian polo-like kinase 1 (Plk1) is critically required for proper M-phase progression. In addition to centrosomes and midbody, Plk1 localizes to late interphase and early mitotic kinetochores in a manner that requires the function of its C-terminal polo-box domain (PBD). However, the underlying mechanism of how Plk1 is recruited to and delocalized from this site and the physiological significance of this dynamic cellular process remain largely uninvestigated. We have previously shown that Plk1 recruits itself to interphase centromeres by phosphorylating and binding to the T78 residue of a kinetochore scaffold protein, PBIP1 (also called MLF1IP and CENP-U/50) through its PBD. PBIP1 also tightly interacts with another centromere component, CENP-Q, and forms a stable complex. Here, we demonstrated that the PBIP1-CENP-Q complex becomes hyperphosphorylated and unstable at the late stage of the cell cycle, and rapidly delocalized from late stage kinetochores as cells enter mitosis. Plk1 phosphorylated the CENP-Q subunit of the PBIP1-CENP-Q complex at multiple sites, and mutation of 9 Plk1-dependent phosphorylation sites to Ala (9A) enhanced CENP-Q association with chromatin and prolonged CENP-Q localization to kinetochores. Conversely, mutation of the 9 sites to phospho-mimicking Asp/Glu (9D/E) residues dissociated CENP-Q from chromatin and untimely delocalized CENP-Q from kinetochores. Strikingly, however, both the 9A and 9D/E mutants induced a defect in mitotic progression and chromosome segregation, suggesting that either prolonged localization to or precocious delocalization from kinetochores can obliterate the CENP-Q function. Notably, cytosolic PBIP1-CENP-Q complex exhibited a greater sensitivity to proteasome-dependent degradation than chromatin-bound complex in a manner that does not require Plk1 activity, suggesting that Plk1-dependent delocalization itself can indirectly lead to the degradation of the complex in mitotic cytosol. Thus, we propose that timely phosphorylation of the PBIP1-CENP-Q complex by Plk1 is important for proper delocalization and ultimate destruction of the complex, and these processes are important not only to promote Plk1-dependent mitotic progression but also to reset the timing of Plk1 recruitment to kinetochores in the next cell cycle.

P1053**Board Number: B380****Mps1 is a critical sensor that coordinates microtubule attachment and spindle checkpoint activation.**Z. Ji¹, H. Gao¹, H. Yu^{1,2};¹Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX, ²Howard Hughes Medical Institute, Dallas, TX

The fidelity of mitotic chromosome segregation is ensured by a surveillance mechanism called the mitotic spindle checkpoint. Generally, it senses the microtubule-unattached kinetochore and generates “wait” signals to arrest cells in mitosis. In turn, proper kinetochore-microtubule (MT) attachment could turn off the checkpoint. However, it is still unclear how the spindle checkpoint is directly tuned by microtubule attachment. Mps1 is an essential checkpoint kinase, whose kinetochore recruitment is a critical step for checkpoint activation. In this study, we have discovered a direct interaction between Mps1 and the outer kinetochore Ndc80 complex (Ndc80C). This interaction is mediated by two independent motifs of Mps1, N-motif and M-motif. Surprisingly, of the two, only N-motif contributes to Mps1 kinetochore localization. M-motif of Mps1, although dispensable for localization, is crucial for substrate phosphorylation and downstream signaling. In addition, Ndc80C is also the kinetochore receptor for spindle microtubules. Strikingly, the Ndc80C-microtubule interaction can directly prevent both N- and M-motif from engaging with Ndc80C, which restricts Mps1-Ndc80C binding and subsequent checkpoint activation to microtubule-unattached kinetochores. Taken together, we conclude that Mps1 is an attachment sensor that is critical to coordinate checkpoint activation and microtubule attachment.

P1054**Board Number: B381****Centromeric Plk1 activity stabilizes the kinetochore to ensure high-fidelity chromosome segregation.**R. Lera¹, M.E. Burkard¹;¹University of Wisconsin-Madison, Madison, WI

Polo-like kinase 1 (Plk1) has a multitude of required functions which are executed during human cell division. In early mitosis, Plk1 localizes to kinetochores, amongst other sites. Over 20 substrates of Plk1 have been described at the kinetochore but their relative contributions to overall mitotic fidelity remain obscure. Here, we used allele-specific chemical probes to isolate Plk1 functions at the kinetochore. With modest reductions in overall Plk1 activity, most functions are preserved, but chromosomes missegregate on bipolar spindles in anaphase. Mechanistically, this occurs due to tension-sensitive kinetochore dysfunction wherein CENP-A and all other kinetochore proteins tested are lost from a single sister chromatid, which is silent to the mitotic checkpoint. Loss of histone-variant CENP-A is particularly surprising because Plk1 is not readily detected at the centromere. To evaluate whether the requisite

Plk1 activity arises indirectly from outer-kinetochore or occurs directly from centromeric activity, we tethered complementing Plk1 kinase domain to distinct locales from microtubule tips (Kif18, MCAK), to kinetochore (Hec1, BubR1, Bub1, Dsn1) to the centromere (MCAK, CENP-A, CENP-B), and to chromatin (H2B). We separately control activity of this complementing kinase using orthogonal chemical genetics (Burkard et al. ACS Chem Biol 7:978, 2012). After confirming localization and activity, we evaluated for ability to rescue the chromosome missegregation phenotype. Surprisingly, only inner-centromeric and chromatin-localized Plk1 activity rescue kinetochore dysfunction (MCAK- and H2B-tethered). Crucially, the rescue is lost with specific chemical inhibition of this localized activity. These two constructs co-localize only at the centromere, suggesting that this is the site of Plk1 action. To test this, we performed MCAK mutational analysis to independently enforce localization at microtubule tips or the centromere. Only Plk1 tethered to MCAK at the centromere was functional. Thus, our data provide convergent evidence of critical mitotic functions of Plk1 that occur at the inner centromere. Our results suggest that a small pool of active Plk1 exists at the centromere, where it is not readily detected. Alternatively, kinetochore-bound Plk1 may phosphorylate centromeric proteins until tension from the spindle effectively removes the possibility of Plk1-centromere overlap. In either case, only a limited pool of Plk1 is available to act at centromeres, explaining why the kinetochore –dysfunction phenotype is highly sensitive to modest losses of kinase activity. We are thus able to isolate and localize discrete Plk1 functions using chemical biology to obtain mechanistic insight into function of this pleotropic mitotic kinase.

P1055

Board Number: B382

Lateral and end-on kinetochore attachments are required for chromosome segregation in *Drosophila* oocytes.

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Proper chromosome segregation is achieved through the regulated interaction of chromosomes with a bipolar array of microtubules that constitute the spindle. The meiotic spindle in the oocytes of many organisms, including humans and *Drosophila*, is built in the absence of the classical microtubule-organizing centers known as centrosomes. In the presence of centrosomes, chromosome segregation depends on interactions between the kinetochore, a protein complex that assembles at the centromere, and microtubules that connect to the centrosomes. However, it has been suggested that kinetochore-microtubule interactions may not participate in chromosome congression or segregation in acentrosomal oocytes. To determine the role of kinetochores during spindle assembly and chromosome orientation in *Drosophila* oocytes, we used a technique in which RNAi knockdown of kinetochore components is confined to oocytes after meiotic entry. Our results suggest that the proportion of kinetochore-dependent microtubules in the spindle changes as oocytes progress from prometaphase to metaphase, such that kinetochores are essential for metaphase spindle stability. In addition, we found that both lateral and end-on kinetochore-microtubule interactions are required for the proper

orientation of centromeres toward spindle poles, although lateral interactions are sufficient for prometaphase chromosome movements. Surprisingly, we also found that the kinetochore may play a role in sister centromere co-orientation, perhaps by affecting sister chromatid cohesion. These results support a model in which oocyte chromosomes initially interact with microtubules laterally at kinetochores while orientation is established, then end-on kinetochore-microtubule interactions are required to direct chromosome segregation.

P1056

Board Number: B383

CLIP-170 is required to recruit PLK1 to kinetochores during early mitosis for chromosome alignment.

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During cell division, faithful chromosome segregation is dependent on the efficient and correct formation of kinetochore–microtubule (KT–MT) attachments on the mitotic spindle, which facilitate the progress of chromosome alignment. Polo-like kinase 1 (PLK1) is a key regulator of mitotic division and required for the formation of stable KT–MT attachments and chromosome alignment. Cytoplasmic linker protein (CLIP)-170, a microtubule plus-end-tracking and outer kinetochore protein, also has a role in KT–MT attachment and chromosome alignment during mitosis. However, the mechanism of CLIP-170's role in chromosome alignment remains unknown. Here we report that CLIP-170 functions in chromosome alignment by recruiting PLK1 to kinetochores during early mitosis and, in turn, stabilizing K-fibers, both of which are mediated by phosphorylation of its CDK1-site T287. We show that CLIP-170 co-localizes with PLK1 at kinetochores during early mitosis. Depletion of CLIP-170 results in a significant reduction of PLK1 recruitment to kinetochores leading to reduction of kinetochore localization of CENP-E and CLASPs. The reduction of localization of kinetochore proteins causes kinetochore-fiber (K-fiber) instability and defects in chromosome alignment at the metaphase plate. These phenotypes are dependent on the phosphorylation of CLIP-170 at a CDK1-site, T287, as ectopic expression of wild-type CLIP-170, but not non-phosphorylatable mutant, CLIP-170–T287A, restores PLK1, CENP-E and CLASPs localization at kinetochores and rescues K-fiber stability and chromosome alignment. Immunoprecipitation experiments show that the interaction between CLIP-170 and PLK1 is dependent on CLIP-170 phosphorylation on the CDK1-site, T287, during early mitosis. These data suggest that CLIP-170 acts as a novel recruiter and spatial regulator of PLK1 at kinetochores during early mitosis, to promote K-fiber stability and chromosome alignment for error-free chromosome segregation. The relative contributions of different pathways for PLK1 recruitment to kinetochores to facilitate KT–MT attachment and chromosome alignment will be an important topic for future studies.

P1057**Board Number: B384****The novel mitotic factor CR1 governs PLK1 docking and function at kinetochores.**H. Lee¹, H. Cho¹;¹Ajou University, Suwon, Korea

Polo-like kinase1 (PLK1) at mitotic kinetochores plays an essential role for faithful chromosome alignment and segregation; however, it remains unknown how PLK1 is recruited to kinetochores. Here, we show that novel mitotic factor CR1 resides at mitotic kinetochores and directly binds PLK1. Kinetochores localization of PLK1 was disrupted by CR1 depletion, which was fully rescued by the C-terminal fragment of CR1. CR1 was phosphorylated by CDK1 at the Ser1375, which triggers PLK1 deposition and subsequent PLK1-mediated phosphorylation on CR1. CR1S1375A neither bound PLK1 nor accumulated PLK1 at kinetochores. Chromosome alignment was profoundly defective in CR1-depleted cells; these defects were rescued by phosphomimetic CR1S1375D. Together, the results indicate that CR1 is an essential centromeric component that recruits PLK1 to kinetochores and plays a crucial role for genomic integrity.

P1058**Board Number: B385****How the kinetochore harnesses microtubule force and centromere stretch to move chromosomes revealed by a FRET tension sensor within Ndc80 protein.**A. Suzuki¹, B.L. Badger¹, J. Haase¹, T. Ohashi², H.P. Erickson², E.D. Salmon¹, K.S. Bloom¹;¹Biology, University of North Carolina, Chapel Hill, NC, ²Department of Cell Biology, Duke University Medical Center, Durham, NC

The Ndc80 complex (Ndc80, Nuf2, Spc24, Spc25) is a highly conserved kinetochore protein essential for end-on anchorage of kinetochore microtubule (kMT) plus-ends and for force generation coupled to attached plus-end polymerization and depolymerization. Spc24/Spc25 at one end of the Ndc80 complex binds the kinetochore. The N-terminal tail and CH domains of Ndc80 at the other end bind microtubules (MTs). An internal loop domain of Ndc80 may be linked to microtubule-associated proteins (MAPs) such as the Dam1 complex. To determine how the MT and MAP binding domains of Ndc80 contribute to force production at kinetochores in budding yeast, we have inserted a characterized FRET tension sensor into Ndc80 protein about halfway between its CH and loop domains. During the cell cycle, the FRET sensor reported low tension from late anaphase through interphase and high tension at metaphase, when pericentromeric chromatin is maximally stretched between sister kinetochores. In addition, we found that both Stu2, which concentrates at MT plus ends during polymerization, and tension reported by the FRET sensor fluctuated over time indicating that tension at the MT binding domains of Ndc80 is different for the polymerization and depolymerization phases of kMT dynamic instability. Surprisingly, FRET sensor measurements showed that reducing MT dynamicity with low dose benomyl at metaphase caused a major drop in tension at the MT binding domains of Ndc80 without loss of normal centromere

stretch. In addition we found that tension at the MT binding domains of Ndc80 is abnormally low with a Dam1 mutation (DAM1-765, Shimogawa et al., 2006) that enhances Dam1 complex affinity for MTs and produces at metaphase hyper-centromere stretch. The above data suggest that Dam1 complex bound to Ndc80 complex at a site inside the FRET tension sensor has a dominant role, compared to the MT binding domains of Ndc80, for attachment to kMT plus ends at metaphase. Based on the above in vivo studies and in silico simulations, we propose a mechanical model for the Ndc80 force coupler at budding yeast kinetochores. During depolymerization, pushing forces from peeling protofilaments against the Dam1 complex pulls on the Ndc80 complex to stretch the centromere and drags the MT binding domains of Dam1 and Ndc80 poleward along their kMT at the velocity of depolymerization until a switch occurs to polymerization. Then, during polymerization, pulling force from centromere stretch on the Ndc80 complex drags the MT binding domains of Dam1 and Ndc80 complexes away from the pole along their kMTs until a switch occurs to depolymerization.

P1059

Board Number: B386

The Nucleoporin MEL-28/ELYS promotes chromosome segregation during meiosis I by recruiting Protein Phosphatase 1 (PP1).

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In many species that undergo open mitoses, a subset of nucleoporins (most prominently the Y-complex, also known as the Nup107/160 complex) localize to kinetochores following nuclear pore disassembly. Despite wide conservation of this dynamic localization pattern, the function of these nucleoporins in chromosome segregation remains poorly understood. We have been using *C. elegans* as a model to understand how the Y-complex functions in chromosome segregation. This effort has revealed a key role for one complex member MEL-28 (ELYS in vertebrates) in meiosis I anaphase. MEL-28 depletion prevents the segregation of chromosomes in oocyte meiosis I, although it does not perturb bipolar spindle formation, spindle positioning, chromosome alignment, or activation of the cohesin-cleaving enzyme separase. Meiotic chromosome segregation activity is intrinsic to MEL-28 and distinct from its role in nuclear pore assembly, as it does not require other Y-complex subunits. MEL-28 is 1784aa in length with the first half forming folded beta-propeller and alpha-solenoid domains and the second half being primarily unstructured. Truncation analysis of MEL-28 revealed a short 50aa region adjacent to the structured domains as being essential for meiotic chromosome segregation. Sequence analysis of this region revealed a potential PP1 (protein phosphatase 1) docking motif and biochemical and yeast 2-hybrid assays confirmed motif-dependent direct interaction of MEL-28 with GSP-1/2 (*C. elegans* PP1 catalytic subunit homologues). Importantly, mutation of the PP1 docking motif phenocopied the MEL-28 depletion phenotype in meiosis I anaphase chromosome segregation. However, in contrast to MEL-28 depletion, the PP1 docking motif mutant did not appear to disrupt nuclear pore assembly, which is enabling current efforts to investigate the function of the MEL-28-PP1 interaction during mitotic chromosome segregation. Notably, despite poor primary sequence conservation in the unstructured

region, the PP1 docking motif is conserved in vertebrate homologues of MEL-28. These results suggest that the MEL-28-PP1 interaction plays a conserved role in chromosome segregation, which has become functionally critical in the context of meiosis I anaphase in *C. elegans* oocytes.

P1060

Board Number: B387

Kn1-dependent Determinants of Human Mad1 Kinetochores Recruitment.

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Several lines of evidence point toward a central role for the protein KNL1 in kinetochore function. KNL1 is necessary for proper kinetochore targeting and function of proteins involved in several mitotic pathways, including checkpoint signaling and checkpoint silencing. In yeast, it has been recently determined that Bub1, recruited to kinetochores through KNL1, is the main receptor for Mad1 and Mad2, the key proteins that signal activation of the mitotic checkpoint. In human cells, however, it is not yet well established which kinetochore protein(s) function as the Mad1/Mad2 receptor. Previous work showed that Bub1 depletion from human cells results in decreased Mad1/Mad2 levels at kinetochores. Interestingly, it has also been proposed that Mad1 localization depends on proper kinetochore recruitment of the RZZ complex, required for checkpoint silencing and also dependent on KNL1, and Zwint-1. Because the ability of KNL1 to mediate Mad1/Mad2 and RZZ targeting to kinetochores sets the foundation for checkpoint signaling, we investigated the KNL1-dependent requirements for Bub1, Mad1, and RZZ complex kinetochore localization, and the dependencies among these proteins. Consistent with data from other organisms, we find that Mad1 localization to kinetochores follows the same KNL1-dependence pattern as that of Bub1 and is significantly decreased upon Bub1 depletion. We also find that depletion of Rod (a subunit of the RZZ complex) results in almost a complete loss of Mad1 from kinetochores. However, a KNL1 fragment that is able to recruit Rod is not sufficient for Mad1 kinetochore localization. Finally, depletion of Bub1 caused a significant decrease in the kinetochore localization of the RZZ complex. Our results provide evidence for a Kn1-dependent hierarchy for Mad1 recruitment to mitotic kinetochores.

P1061**Board Number: B388****Kif18a stabilizes kinetochore microtubule attachments.**H. Kim¹, A. Czechanski², C. Byers², I. Greenstein², C. Fonseca¹, L. Reinholdt², J.K. Stumpff¹;¹Molecular Physiology and Biophysics, University of Vermont, Burlington, VT, ²Genetic Research Science, Jackson Laboratory, Bar Harbor, ME

During mitosis, duplicated genetic material is segregated to each daughter cell, mediated by the dynamic network of microtubules within spindle. A specialized protein complex known as the kinetochore provides the physical linkage between the chromosome and the spindle microtubules. The phosphorylation state of kinetochore proteins influences their affinity for the microtubule lattice. Stable, high-affinity attachments between microtubules and kinetochores maintain the structural integrity of the spindle, are required to silence the spindle assembly checkpoint, and facilitate chromosome segregation. The mitotic kinesin Kif18a (kinesin-8) has been shown to regulate kinetochore microtubule (k-fiber) length to promote chromosome alignment (Mayr et al, 2007, Stumpff et al, 2008). However, our data suggest that Kif18A also functions to stabilize kinetochore-microtubule attachments. Somatic human cells depleted of Kif18A display an increase in the number of kinetochores that are positive for MAD1, a key component of the spindle assembly checkpoint. In addition, depleting Kif18A from diploid human cells also leads to a modest increase in the time required for cells to progress from nuclear envelope breakdown to anaphase. Furthermore, we show that primordial germ cells, but not somatic cells, from Kif18A mutant mice arrest in mitosis and display a severe proliferation defect. These data indicate that Kif18A promotes connections between kinetochores and microtubules and that this function may be essential during mammalian development for the proliferation of germ line precursors.

P1062**Board Number: B389****A role for Aurora A kinase in kinetochore-microtubule attachment regulation.**K.F. DeLuca¹, J. Mick¹, J.G. DeLuca¹;¹Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO

One of the most important regulatory aspects of chromosome segregation is the ability of kinetochores to precisely control their attachment strength to microtubules. During early mitosis, attachments are labile to prevent prematurely stabilized connections, whereas during late mitosis, attachments are stabilized so that forces can be generated for chromosome congression and to silence the spindle assembly checkpoint. Central to this regulation is Aurora B kinase, a mitotic kinase that phosphorylates kinetochore substrates to promote microtubule turnover. Arguably, one of the most critical Aurora B targets for this regulation is the Hec1 subunit of the NDC80 complex, which is the primary force-transducing link between kinetochores and microtubules. Phosphorylation of the unstructured N-terminal tail of Hec1 reduces NDC80-microtubule affinity in vitro and kinetochore-microtubule affinity in cells. Previous work using phospho-specific antibodies to multiple Hec1 tail target sites revealed high

levels of phosphorylation in early mitosis and low levels in late mitosis, consistent with the idea that high levels of Hec1 phosphorylation promote rapid kinetochore-microtubule turnover. Recently, we generated a phospho-specific antibody against Serine 69 of the Hec1 tail to determine whether this residue, positioned close to the Hec1 calponin homology domain (which directly binds the microtubule lattice), exhibits a similar phosphorylation pattern to that of other tail phospho-residues. Strikingly, the phosphorylation pattern for Ser69 is quite different from that of Ser8, Ser15, Ser44, and Ser55, as Ser69 phosphorylation is high during early mitosis and does not decrease during metaphase. In addition, unlike the other phospho-sites, the pSer69 signal is not significantly reduced following Aurora B kinase inhibition. Testing other mitotic kinase inhibitors led us to Aurora A kinase, whose inhibition in PtK1, HeLa, and RPE1 cells results in a significant decrease in the phosphorylation of Hec1-Ser69, but not of other Hec1 residues. Furthermore, we found that inhibition of Aurora A kinase prevents proper metaphase chromosome dynamics. These findings uncover an unexpected role for Aurora A kinase, as it is known to localize to spindle poles and is primarily implicated in spindle assembly.

P1063

Board Number: B390

Stable kinetochore-microtubule attachment satisfies the spindle assembly checkpoint in the absence of sister kinetochore biorientation.

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Accurate segregation of replicated chromosomes during mitosis is essential for the maintenance of genomic integrity. To ensure faithful chromosome segregation, eukaryotic cells have developed a surveillance network called the spindle assembly checkpoint (SAC) that delays anaphase onset until sister kinetochores of duplicated chromosomes are properly attached to microtubules emanating from opposite spindle poles. SAC proteins accumulate at kinetochores of mitotic chromosomes in the absence of stable microtubule attachments and transmit a signal that inhibits the Anaphase Promoting Complex/Cyclosome (APC/C). Once microtubules attach to kinetochores, SAC proteins are evicted, which leads to silencing of the SAC inhibitory signal, activation of the APC/C, and subsequent mitotic exit. Precisely how the inhibitory SAC signal is extinguished in response to microtubule attachment remains unresolved, although it is likely that both the physical attachment of microtubules to kinetochores and the ensuing pulling forces that result are important aspects of the signaling process. Here we investigate how hyper-stabilization of kinetochore-microtubule attachment affects progression through mitosis. To induce kinetochore-microtubule hyper-stabilization, we used a mutant version of the kinetochore-microtubule attachment factor Hec1 that is unable to be phosphorylated by Aurora B kinase: 9A-Hec1. Specifically, this is a mutant in which nine identified Aurora B target sites were mutated to alanine (9A). Cells depleted of endogenous Hec1 and rescued with 9A-Hec1-GFP harbor hyper-stable kinetochore-microtubules as evidenced by increased inter-kinetochore distances, increased kinetochore-fiber size, an accumulation of erroneous attachments including merotelic and syntelic, and defects in correcting such errors. We find that the hyper-stable kinetochore-microtubule attachments in cells expressing 9A-Hec1

GFP are sufficient to silence the SAC, even in the absence of chromosome bi-orientation or experimentally induced tension. Furthermore, we find that hyper-stable kinetochore-microtubules are sufficient to induce structural changes in kinetochores, as evidenced by development of intra-kinetochore stretch, even in the absence of amphitelic attachment. Our results suggest that the structural changes brought on by stable kinetochore-microtubule attachment are sufficient to silence the SAC, and furthermore, these changes are sensed autonomously by individual kinetochores.

Spindle Assembly 2

P1064

Board Number: B391

The Missing Link in Kinetochore-Microtubule Error Correction: Detachment at Spindle Poles.

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Faithful DNA segregation between dividing cells requires chromosomes to attach to microtubules from opposite spindle poles (bi-orientation). This process is regulated by Aurora B kinase, which selectively destabilizes microtubule attachments until proper orientations are achieved. To correct syntelic attachments, in which sister kinetochores are attached to the same spindle pole, Aurora B drives the mal-oriented chromosome towards the pole without releasing its microtubule attachments. The chromosome then re-congresses and aligns the metaphase plate, but it is unclear how erroneous microtubules are detached before proper attachments are formed. Here we demonstrate that proximity to the spindle poles destabilizes kinetochore-microtubules. To study the pole effect without interference from other components of error correction, we used meiotic systems featuring asymmetrical chromosomes (i.e. trivalents, unbalanced bivalents) that are off-centered even when correctly attached. We show that kinetochores near the spindle poles are less likely to have stable microtubule attachments and that these attachments can be restored by inhibiting Aurora A kinase. We also show that MAD1 accumulation on kinetochores is directly linked to the poleward movement of chromosomes. Our results suggest that syntelic attachments can be destabilized by pole proximity, which is critical for ensuring chromosome bi-orientation and accurate segregation.

P1065**Board Number: B392****Generational conflict in mitosis: old centrosome retains non separated sister chromatids.**I. Gasic¹, J. Meadows², P. Nerurkar³, P. Meraldi¹;¹Cell Physiology and Metabolism, University of Geneva, Geneva, Switzerland, ²Biomedical Cell Biology, University of Warwick, Coventry, UK, ³Institute of Biochemistry, ETH Zürich, Zurich, Switzerland

The centrosomes of the two spindle poles contain centrioles of different age, one old and one young, creating an inherent asymmetry in the mitotic spindle. In the self-renewing asymmetric stem cell divisions, unequal old-young centrosome behaviour results in a bias in centrosome positioning and inheritance, which is critical for daughter cell fate determination. In contrast, it is assumed that in symmetric cell divisions the behaviours of the two centrosomes are indistinguishable. Here, we demonstrate that even in somatic symmetric cell divisions the old and the young centrosomes are functionally different during mitosis. Both in transformed and non-transformed human cells centrosomes display a difference in the stability of kinetochore-microtubules associated to them. This difference is reflected in the efficiency of chromosome alignment onto the metaphase plate, as we find that chromosomes bound to the old centrosome are delayed in their alignment. The unequal stability of kinetochore-microtubules persists in anaphase: merotelic sister chromatids that fail to separate have a 9-fold higher probability to mis-segregate to the cell that inherits the old centrosome. This bias in chromosome mis-segregation is conserved during evolution, as non-separated merotelic sister chromatids have the same 9-fold higher probability to end up in the cell with the old spindle pole body in the fission yeast *Schizosaccharomyces pombe*. We thus postulate that the inherent age difference of centrosomes favours stem cells to retain chromosomes in the event of sister chromatids non-separation.

P1066**Board Number: B393****Scaling and Evolutionary Dynamics of the Mitotic Spindle.**R. Farhadifar^{1,2}, C.F. Baer³, A. Valfort⁴, E. Andersen⁵, T. Müller-Reichert⁶, M. Delattre⁴, D.J. Needleman^{1,2,7};¹FAS Center for Systems Biology, Harvard University, Cambridge, United States, ²Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA, ³Dep. Biology, University of Florida, Gainesville, FL, ⁴CNRS, Lyon, France, ⁵Molecular Biosciences, Northwestern University, Evanston, IL, ⁶Medical Faculty "Carl Gustav Carus", TU Dresden, Dresden, Germany, ⁷School of Engineering and Applied Sciences, Harvard University, Cambridge, MA

Cellular structures such as the nucleus, the Golgi apparatus, centrioles, and the spindle show remarkable diversity across different species. The mechanisms that produce these variations in cell biology are not known. We study the mechanisms that contribute to variations in morphology and dynamics of the

mitotic spindle, the subcellular structure that segregates the chromosomes during cell division. We developed a high-throughput microscopy platform and automated image analysis software that allows us to obtain quantitative information on the structure and dynamics of spindles from thousands of embryos in hundreds *C. elegans* lines. We found extensive variations among wild isolates of *C. elegans* for all traits we studied including: cell size; the length, motion, and speed of elongation of the spindle; the size of centrosomes; the positioning of the cleavage plane. We demonstrate that the effects of spontaneous mutations on cell division in a single generation, combined with stabilizing selection on embryo size, are sufficient to quantitatively explain both the levels of within species variation in the spindle and the diversity in the spindle over ~100 million years of evolution. Our results argue that selection acts predominantly on embryo size, which is also the size of the cell for the first mitotic division in nematodes, and indirectly influences the spindle morphology and structure through the scaling of the spindle with cell size. Our finding of extensive within species variation for the spindle demonstrates that there is not just one “wild type” form, rather cellular structures can exhibit a surprisingly broad diversity of naturally occurring behaviors. Also, mapping the genetic basis of these within species differences opens a novel route for studying evolutionary and mechanistic aspects of cell biology.

P1067

Board Number: B394

Homology modeling and functional analysis of the mitotic checkpoint complex in budding yeast.

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The interactions of Cdc20p/Mad3p/Mad2p, members of the mitotic checkpoint complex (MCC) in budding yeast, were examined using computational chemistry and cellular biology. Computer analysis of target protein structure was compared with observational data from *in vivo* studies. The focus of computer modeling was to evaluate structure, conformation and functional domains, specifically the KEN box receptor and D box receptors, sites of Cdc20/Mad3 interaction. Furthermore, the effects of known point mutations in CDC20 on the complex were also studied. To facilitate computational analysis, models of wild type Cdc20p, Mad3p, and Mad2p were constructed through homology modeling. The crystal structures of the *Schizosaccharomyces pombe* (fission yeast) homologs were used as the templates to generate homology models. Sequence alignment and manual optimization was performed in SWISS-MODEL and homology modeling completed in Molecular Operating Environment (MOE). Homology models were analyzed for accuracy by comparing side chain positions to crystal structures and structurally validated via Molprobit. Molecular dynamics (MD) simulations were then carried out in Amber10 for gross protein minimization and data acquisition of protein behavior in solution. Visual Molecular Dynamics (VMD) was used for frame-by-frame analysis of the system for evaluation of intermolecular bonding, protein-solvent interactions and movements. PyRosetta was used to refine average MD structures through a series high- and low-resolution conformational sampling functions.

Docking of the three MCC proteins was also performed through PyRosetta.

Additional analysis was conducted to look at the effect of the *cdc20-1* mutation on functional domains in Cdc20, targeting KEN and D box receptors, and to evaluate potential interference with Mad3 binding. Homology models were studied at temperatures simulated to match the permissive and non-permissive temperature for the *cdc20-1* mutant.

To complement the computational studies of interactions within the MCC complex, the *in vivo* localization of MCC proteins such as Cdc20p and Mad3p was examined using GFP tagging and fluorescence microscopy. The version of the protein expressed in the *cdc20-1* mutant was also studied and compared to the wild type.

The main objective of the *in vivo* studies was to understand the difference between the normal MCC protein interactions and the *cdc20-1* mutant protein when it is a part of the MCC. Our goal was to better understand interactions among members of the MCC complex; information that could lead to an enhanced understanding of mitosis in other organisms including humans.

P1068

Board Number: B395

Isolation of the tension-mediated spindle assembly checkpoint response using a novel Taxol-sensitive yeast model.

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The spindle assembly checkpoint (SAC) delays anaphase until all kinetochores bind microtubules, and there is tension between sister chromatids, which signifies bipolar attachment. Despite its critical role in maintaining genomic stability, mechanistic details of the tension-mediated SAC response remain unclear. One significant limitation of current model systems is the experimental intractability of the tension and the attachment status at kinetochores. In metazoan cells, Taxol stabilizes microtubules and reduces tension, but also causes a subset of kinetochore sites to remain unbound by microtubules. Budding yeast has been a preeminent system for investigating SAC function, but a major limitation is that microtubule stabilizers like Taxol have no effect on yeast cells. Conventionally, kinetochore tension is reduced by inhibiting chromosome replication or cohesion. However, these treatments stimulate kinetochore detachment and also disturb bipolar spindle organization. We developed a novel, Taxol-sensitive yeast system in which we can limit tension across sister chromatids independent of microtubule detachment. This allowed us to experimentally isolate a tension-specific SAC response in otherwise normal, bipolar spindles. By manipulating the cell cycle we allowed proper bipolar spindles to assemble in the absence of Taxol. We observed that, similar to metazoan cells, addition of Taxol decreased the distance between sister kinetochores. Significantly, yeast kinetochores bind only one microtubule, and the reduced tension did not disrupt bipolar attachment. Strikingly, Taxol induced a transient SAC-dependent delay in anaphase onset. Similarly, while deletion of all SAC proteins makes cells supersensitive to microtubule destabilizing drugs, a subset of deletions rendered cells supersensitive to Taxol, suggesting that attachment and tension sensing may have different signaling

requirements. In summary, we developed and leveraged a novel model system to demonstrate a distinct, tension-mediated SAC response.

P1069

Board Number: B400

Three-dimensional reconstructions of the first mitotic spindle reveals a novel mechanism for spindle assembly in *C. elegans*.

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Upon entering mitosis, the microtubule cytoskeleton of any eukaryotic organism undergoes a fundamental reorganization to form a bipolar spindle, which first aligns the condensed chromosomes on the metaphase plate and then segregates them to the resulting daughter cells. Despite the fact, that bipolar spindles are composed of the same building blocks and conduct the same function, there is a huge variety in spindle size, assembly and architecture across eukaryotic species. In order to understand spindle architecture, it is essential to understand the detailed 3D ultrastructure of a mitotic spindle and to quantitatively study the key features. We generated full 3D reconstructions of the first mitotic spindle in *C. elegans* during metaphase and anaphase using serial-section electron tomography. We combined this detailed structural data with dynamic data from light microscopy. Using this approach we were able to detect individual microtubules attached to the holocentric kinetochores. This analysis provided us with detailed information on the number of microtubules attached per chromosome, as well as the position of attachment sites on the chromosomes. To our surprise the percentage of kinetochore microtubules within the spindle is very small, only about 5%. In contrast to our expectations, our results argue that kinetochore microtubules do not originate at the centrosomes, but polymerize from kinetochores, most likely with the minus ends of the microtubules facing towards the centrosome. This suggests a yet unknown mechanism in *C. elegans*, in which microtubules nucleated from centrosomes interact with microtubules originating from kinetochores to form the bipolar mitotic spindle.

P1070

Board Number: B401

Polar ejection forces are involved in Spindle Assembly Checkpoint satisfaction during mitosis of unreplicated genome.

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Complete DNA replication during S phase and establishment of bipolar attachments during mitosis is from crucial importance for the life of the cell. In our work we are investigating the Spindle Assembly

Checkpoint (SAC) satisfaction in the absence of interkinetochore tension. For that propose, we used Dup (double parked) RNAi depletion, a protein involved in origin of replication and post-replication checkpoint response at the end of G2 phase, which allowed *Drosophila* S2 cells to enter mitosis without previous replication of the genome. This interesting phenomenon is known as mitosis of an unreplicated genome (MUG). S2 cells undergoing MUG have condensed single chromatids, unstably attached to spindle microtubules. By using live cell imaging and *Drosophila* S2 cells stably expressing fluorescently labeled proteins we were able to follow the dynamics of mitotic proteins during MUGs. *Drosophila* S2 cells undergoing MUG stayed longer time in mitosis compared with control cells, which was result of slower cyclin B degradation in RNAi treated cells. Nevertheless, after delay (2-3h) Dup depleted cells were able to exit mitosis with previous SAC satisfaction.

The correct mechanism how SAC can be satisfied in the absence of interkinetochore tension is unknown. Here we show the role of Polar Ejection Forces (PEFs) in establishment of intrakinetochore stretch, as a mechanism for SAC satisfaction in the absence of interkinetochore tension during mitosis of unreplicated genome.

P1071

Board Number: B402

Measurement of the force that centers the mitotic spindle in the early *C. elegans* embryo using magnetic tweezers.

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Because the plane of cell division bisects the mitotic spindle, the positioning of the spindle specifies the size and location of the two daughter cells. Little is known, however, about the mechanical processes underlying spindle positioning. To study this mechanism, we applied calibrated magnetic forces to the spindle via super-paramagnetic beads inserted into the cytoplasm of one- and two-cell *C. elegans* embryos.

At metaphase in the one-cell embryo, a 20 pN force displaced the mitotic spindle pole of one-cell embryos approximately 1 μm away from the anterior-posterior axis over 10-20 seconds. By tracking the bead displacement, we found that the spindle behaved roughly as a damped spring with a spring constant of 18 ± 12 pN/ μm and a drag coefficient of 127 ± 65 pN·s/ μm (mean \pm SD).

The centering stiffness was two-fold higher in the two-cell embryo, consistent with a centering mechanism that scales inversely with cell size. The stiffness increased when the number of microtubules reaching the cortex was increased two fold using RNAi against the depolymerizing kinesin klp-7, but remained roughly the same when the interaction time of microtubules with the cortex was increased using RNAi against efa-6, a cortical catastrophe factor and when cortical pulling forces were reduced by RNAi against gpr-1/2, an activator of the cortical force generators. Furthermore, the stiffness was five-

fold higher during anaphase in both the one- and two-cell stage embryos, indicating that the centering forces change during the cell cycle.

Taken together, our results constrain molecular models of centering. The *gpr-1/2* RNAi knockdown results rule out a role for cortical forces pulling on the spindle via astral microtubules and the scaling with cell argues against pulling by cytoplasmic factors. On the other hand, the results are consistent with centering being mediated by astral microtubules pushing against the cortex as the centering forces scales with the number of microtubules, the cell size and the pushing time of microtubules against the cortex.

Moreover, these results help us to understand how change in mechanical properties is linked to the function of the spindle in metaphase and anaphase.

P1072

Board Number: B403

The equatorial position of the metaphase plate ensures symmetric cell divisions.

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Chromosome alignment in the middle of the bipolar spindle is a hallmark of metazoan cell division. When we force human cells to build asymmetric spindles by creating an asymmetric distribution of centrioles numbers at each spindle pole, we find that they always re-center the position of the metaphase plate before anaphase onset. This plate centering mechanism is made possible by delaying satisfaction of the spindle assembly checkpoint, to provide cells enough time to correct metaphase plate position before anaphase onset. Cells with only one centriole at each pole do not elicit a checkpoint response, indicating that the spindle assembly checkpoint response depends on the asymmetry of centriole distribution, and not on the change in centriole numbers per se. The checkpoint response in cells with an asymmetric centriole distribution is not elicited by unattached or tension free kinetochores, but by minor defects in the maturation of kinetochore-microtubule attachments, which arise as a consequence of an imbalance in microtubule stability between the two half-spindles. Stabilizing these attachments by depleting the microtubule depolymerases KIF2a and MCAK satisfies the checkpoint, and results in anaphase entry with asymmetric spindles that lead to asymmetric cell divisions. We thus postulate that the symmetric metaphase plate position plays an essential role for the control of cell division symmetry.

P1073**Board Number: B404****Theory of spindle assembly.**S. Fürthauer^{1,2}, M. Shelley³, D.J. Needleman^{4,5,6};¹Courant Institute of Mathematical Sciences, New York, NY, ²Harvard University, Cambridge, CT,³Courant Institute, New York University, New York, NY, ⁴FAS Center for Systems Biology, HarvardUniversity, Cambridge, United States, ⁵School of Engineering and Applied Sciences, Harvard University,Cambridge, MA, ⁶Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA

We develop a comprehensive theoretical model for the *Xenopus* meiototic spindle based on the physics of active liquid crystals. Our model captures the interplay between motion caused by molecular scale force generators - such as dyneins and kinesins - and the dynamics of regulatory proteins - such as Ran and its binding partners - which are believed to spatially regulate microtubule nucleation. We recover much of the known phenomenology of spindle assembly and can reproduce its response to external perturbations, which provides insights into the mechanisms responsible for determining spindle shape.

P1074**Board Number: B405****Balanced Activity of Three Mitotic Motors Is Required for Bipolar Spindle Assembly and Chromosome Segregation.**R.G. van Heesbeen¹, M.E. Tanenbaum², R.H. Medema³;¹Division of Cell Biology, Netherlands Cancer Institute, Amsterdam, Netherlands, ²Department of Cellularand Molecular Pharmacology, University of California, San Francisco, San Francisco, CA, ³Division of Cell

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Bipolar spindle assembly requires force to organize the microtubule network. Here, we show that three motor proteins, namely Eg5, Kif15, and dynein, act together to produce the right force balance in the spindle. Excessive inward force results in monopolar spindle formation, while excessive outward force generation results in unstable spindles with splayed spindle poles. Blocking activity of all three motors prevents bipolar spindle formation, but established bipolar spindles are refractory to loss of all motor activity. Further analysis shows that although these preformed spindles remain bipolar, outward force generation is required to establish sufficient tension on kinetochores and to accomplish successful chromosome segregation. Together, these results show how Eg5, Kif15, and dynein work together to build a bipolar spindle and reveal an important role for antagonistic motors in chromosome segregation.

P1075**Board Number: B406****Biophysics of mitotic spindle positioning in *C. elegans* embryos.**H. Wu^{1,2}, C. Yu^{2,3}, R. Farhadifar^{2,4}, D.J. Needleman^{2,3,4};¹Physics, Harvard University, Cambridge, MA, ²FAS Center for Systems Biology, Harvard University, Cambridge, United States, ³School of Engineering and Applied Sciences, Harvard University, Cambridge, MA, ⁴Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA

During cell division, the spindle apparatus, which consists of microtubules, segregates chromosomes and determines the plane of cleavage furrow formation. The mitotic spindles in eukaryotic cells have to be positioned and orientated correctly in specific ways to help cells divide properly. Our goal is to explore the biophysics of mitotic spindle positioning. We choose the *Caenorhabditis elegans* early-stage embryo, which is a powerful tool for studying cell division, as an animal model. During the first mitosis of a *C. elegans* embryo, the spindle elongates and takes chromosomes apart. Meanwhile, the spindle moves off-center giving rise to two daughter cells with different sizes and fates later (called "asymmetric cell division"). We want to know what molecular forces are involved in the positioning and orientating process, and how those forces are coordinated. In vitro experiments have demonstrated the existence of several molecular forces, and different models have been proposed to explain this dynamic process, but it remains unclear which of these possible mechanisms contribute in vivo. In addition, the off-center positioning of the mitotic spindle is accompanied with transverse oscillating motion of two spindle poles. This oscillation effect further opens a window for testing different models. Many issues are still under dispute. First, is it cytoplasmic force or cortical force, or a combination of both, contributing to the positioning of the mitotic spindle? To answer the question, we want to analyze the relative motion of cytoplasmic fluid and microtubules. We use fluorescent nanodiamonds with non-targeting surface treatment as seeding particles. We microinject those nanoparticles into the syncytial gonads of the tubulin:GFP-expressing *C. elegans*, and those particles would be integrated into embryos later. Those fluorescent nanodiamonds can be imaged and tracked, while microtubules can be visualized by GFP-labeled tubulins at the same time during the first mitosis. Analysis of the relative motion helps find out the force sources of spindle positioning. Second, we want to investigate how different forces are coordinated to yield the spindle positioning and orientating behavior. We study the phenomena by perturbing the oscillating motion of spindle poles using laser ablation. For this purpose, we set up a microscopic system to ablate part of the spindle structure with high spatial and temporal precision, and analyze various perturbed motions quantitatively. Combining the above studies, we hope to understand this fascinating dynamic system.

P1076**Board Number: B407****Microtubule Polarity and Biophysics of Anaphase B.**C. Yu¹, H. Wu^{2,3}, R. Farhadifar^{3,4}, D. Needleman¹;¹School of Engineering and Applied Sciences, Harvard University, Cambridge, MA, ²Physics, Harvard University, Cambridge, MA, ³FAS Center for Systems Biology, Harvard University, Cambridge, United States, ⁴Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA

In anaphase B, the spindle elongates by a process that is thought to be driven by the recruitment of proteins to anti-parallel microtubules. However, it remains unclear how the interplay between anti-parallel microtubules and protein localization produces the changes in microtubule behaviors that drive spindle elongation. To study the mechanisms of anaphase B, we have developed a method to quantitatively measure the polarity of microtubules in spindles in real time using a combination of second harmonic generation and two-photon fluorescence microscopy of labeled microtubules. We are combining measurements of the dynamics of microtubule polarity in the spindle with measurements of protein localization and genetic, biochemical, and physical perturbation. We are interpreting our results with the aid of mathematical models and numerical simulations. This work will provide insight into the process by which proteins are recruited to anti-parallel microtubules, the manner in which these proteins modify microtubule behaviors, and the mechanism by which these changes in microtubule behaviors drive spindle elongation, allowing us to test proposed models of anaphase B.

P1077**Board Number: B408****Antiparallel microtubules bridge sister kinetochores and regulate the force balance in the mitotic spindle.**J. Kajtez¹, A. Solomatina¹, I. Šumanovac Šestak¹, M. Novak², N. Pavin², I.M. Tolic^{1,3};¹Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany, ²Department of Physics, Faculty of Science, University of Zagreb, Zagreb, Croatia, ³Dept. Molecular Biology, Ruder Boškovic Institute, Zagreb, Croatia

Accurate segregation of the genetic material in a cell relies on complex molecular machinery called mitotic spindle. Mitotic spindle is a dynamic self-assembled structure that maintains its architectural steady state through accurate balance of forces. Current model of the spindle architecture recognizes two distinct populations of microtubules (MTs) that do not interact with each other: k-fibers and interpolar MTs. According to this model, k-fibers attached to bioriented sister kinetochores interact only through the centromeric region of the chromosome that acts as a spring between the two k-fibers. Here we show that there exists subpopulation of antiparallel MTs named bridging MTs (bMT) that span the region under sister kinetochores and facilitate a lateral connection between corresponding k-fibers. We observed bMT fibers in live-cell fluorescence imaging of HeLa cells. To confirm bMT fiber attachment to k-fibers, we developed a laser ablation assay: the outermost k-fiber is severed to observe movement,

directed away from the spindle center, of the kinetochore pair along with the intact k-fiber and the severed k-fiber stub. Corresponding bMT fiber accompanied this motion verifying that it is laterally attached to opposing k-fibers of one chromosome. In order to test if the forces in the spindle can be explained including the bMT fiber, a theoretical model was constructed. The model accurately predicts the convex shape of the spindle and outer position of kinetochore pair with respect to the corresponding bMT fiber. The model also predicts that thicker bMT fibers generate higher compressive forces in the spindle. To confirm this prediction we created thicker bMT fiber by overexpressing tubulin and antiparallel MT crosslinking protein PRC1. This resulted in much faster movement after the ablation, confirming that compressive force in the spindle was increased. Finally, our results indicate that bMT fiber has an important role as a safety structure for the aberrant mitotic spindle. If the severed k-fiber stub does not reestablish connection with the spindle pole before the onset of anaphase, bMT fiber acts as a rail along which the sister chromatids are moved apart providing an alternative mechanism for chromosome segregation.

P1078

Board Number: B409

Force on spindle microtubule minus ends moves chromosomes.

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The spindle is a dynamic self-assembling machine that coordinates mitosis. The spindle's function depends on its ability to organize microtubules into poles and maintain pole structure despite mechanical challenges and component turnover. Although we know that dynein and NuMA mediate pole formation, our understanding of the forces dynamically maintaining poles is limited: we do not know where and how quickly they act or their strength and structural impact. Using laser ablation to cut spindle microtubules, we identify a force that rapidly and robustly pulls severed microtubules and attached chromosomes poleward, overpowering opposing forces and repairing spindle architecture. Molecular imaging and biophysical analysis suggest that transport is powered by dynein pulling on minus ends of severed microtubules. NuMA and dynein/dynactin are specifically enriched at new minus ends within seconds, re-anchoring minus ends to the spindle and delivering them to poles. This force on minus ends represents a newly-uncovered chromosome transport mechanism that is independent of plus end forces at kinetochores and is well-suited to robustly maintain spindle mechanical integrity.

P1079**Board Number: B410****Kinetochores-microtubule depolymerization opposes Eg5-mediated outward forces in the metaphase spindle.**A.S. Gayek¹, R. Ohi¹;¹Cell and Developmental Biology, Vanderbilt University Medical Center, Nashville, TN

The mitotic spindle requires a balance of forces to maintain its bipolar geometry and proper steady state length. These two features are essential for the spindle to carry out its function of segregating duplicated chromosomes into two daughter cells. The nature of force-balance in the metaphase spindle is still poorly understood. It is well appreciated that kinesin-5/Eg5 slides apart antiparallel microtubules (MTs) from the two centrosomes to push the poles outward as the spindle forms, but the role of Eg5 and Eg5-opposing forces at metaphase is less clear. Eg5-opposing, inward force is thought to come chiefly from MT sliding by minus end-directed motors (dynein and HSET/Kinesin-14), but our data suggest an important role for MT dynamics in generating inward force. We have shown that the ability of spindles to maintain bipolarity without Eg5 depends on the stability of their kinetochore-MTs (K-MTs), a class of MTs that are bundled and attached to chromosomes at the kinetochore whose stability is generally higher in transformed cells than in nontransformed cells. In several transformed cell lines, inhibition of Eg5 at metaphase does not grossly disrupt force-balance, and spindles maintain bipolarity. This maintenance of bipolarity depends on the high stability of their K-MTs. In addition, we have shown that bipolar spindles of nontransformed cells tend to collapse following Eg5 inhibition, but that stabilizing their MTs (including K-MTs) allows them to maintain bipolarity. This demonstrates the importance of K-MT stability for metaphase force-balance. Moreover, expression of a mutated kinetochore protein that increases the strength of kinetochore-to-MT binding exacerbates spindle collapse. The results of this very specific, localized perturbation are consistent with the idea that increasing the strength of kinetochore binding to the MT increases the productive pulling force from each depolymerizing MT, and suggests that plus-end depolymerization of K-MTs provides a significant inward force in the mitotic spindle of nontransformed human cells. This represents a previously understudied mechanism to generate Eg5-opposing, inward forces. Work is ongoing to determine the relative contributions of K-MT depolymerization and minus end-directed motors to force-balance in the metaphase spindle of nontransformed cells.

P1080**Board Number: B411****Contractility of Microtubule Networks in *Xenopus* Egg Extracts.**P. Foster¹, D. Needleman^{1,2};¹School of Engineering and Applied Sciences and FAS Center for Systems Biology, Harvard University, Cambridge, MA, ²Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA

Spindles are self-organized subcellular structures that segregate chromosomes during cell division. Microtubule nucleation, microtubule polymerization dynamics, and motor mediated interactions between microtubules are all crucial for spindle self-organization. We are studying stabilized microtubules in *Xenopus* egg extracts to gain insight into microtubule interactions decoupled from nucleation and polymerization. We have found that networks of stabilized microtubules in *xenopus* egg extracts undergo a dynein-dependent bulk contraction. We are investigating the mechanism of this contraction through quantitative experiments and molecular perturbations. Our finding that microtubule motor interactions are net contractile, not extensile, as is often assumed, has important implications for models of spindle assembly.

P1081**Board Number: B412****Follow the leader: necessity for a leading microtubule assembly pathway during spindle assembly.**T. Cavazza^{1,2}, P. Malgaretti³, N. Brouwers¹, I. Peset⁴, I. Vernos^{1,5};¹Centre for Genomic Regulation - CRG, Barcelona, Spain, ²Universitat Pompeu Fabra, Barcelona, Spain, ³Max Planck Institute for Intelligent Systems, Stuttgart, Germany, ⁴Cancer Research UK, Cambridge Institute, Cambridge, UK, ⁵ICREA, Barcelona, Spain

The mitotic spindle is made of microtubules originated by the centrosomes and by the chromatin pathway. The relative contribution of these different pathways varies depending on the organism, the cell type and the developmental stage. The two pathways differ in topology, timing and kinetics. How these differences are integrated and kept in balance to ensure efficient spindle assembly is currently unclear.

Combining experiments in *Xenopus laevis* egg extract and in tissue culture cells we have addressed this question by changing this balance and examining the consequences on bipolar spindle formation. Decreasing the efficiency of the centrosome in generating microtubules has two main consequences depending on the system: in egg extract, centrosome positioning and therefore inheritance is randomized but the bipolar spindle forms efficiently; in cells, bipolar spindle assembly is compromised. These data suggest that dominant centrosome microtubule activity in cells entering mitosis is essential to facilitate proper spindle assembly. Altogether our data suggest that the two pathways do not act in a cooperative manner, rather we propose that one pathway should lead spindle assembly.

Using mathematical models we show that the two pathways are connected and balanced through a competition for free tubulin. This is particularly relevant in somatic cells in which tubulin is a limiting factor. Our work suggests that tubulin availability acts as a rate-limiting factor that defines the relative contributions to spindle assembly of the centrosomal and of the chromosomal pathways. Depending on the tubulin availability one pathway or the other will lead spindle assembly.

P1082

Board Number: B413

Live-cell imaging of Spindle Assembly Checkpoint dynamics in Germline Stems Cells of intact *C. elegans* larvae and adults.

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The spindle assembly checkpoint (SAC) system inhibits mitotic progression and anaphase onset until all kinetochores are under bi-lateral attachment to microtubules. To date, our understanding of the SAC stems from work in cell culture and in embryos. How the SAC functions *in vivo*, in the varying physiological context of a maturing tissue, is largely unknown. Proteins of the Mitotic Checkpoint Complex bind to the Anaphase Promoting Complex (APC) co-factor cdc20, blocking APC activity. Once the SAC is satisfied, APCcdc20 targets Cyclin-B and securin for proteasome-mediated degradation, allowing separate to cleave the cohesion complex and anaphase to progress. While the SAC was widely viewed as an all-or-none signal, several studies have recently shown that in cell culture, the SAC may function in a graded manner, rather than as a thresholded on-off system. Here, we have developed a live-cell imaging assay to monitor APC activity relative to mitotic progression in germline stem cells (GSCs) within the intact gonad of larval and adult *C. elegans*. We used H2B::mCherry fluorescence variance as a readout of mitotic progression and measure degradation of Cyclin-B::YFP as an indication of APC activity. We validated this assay by activating the SAC and by compromising the SAC and the APC, and observing the change in Cyclin-B degradation rate. We have found that the duration of mitosis is shorter in larval GSCs undergoing expansion of the stem cell pool as compared to older adult GSCs engaged in stem cell homeostasis. In all cases, we have found that the rate of Cyclin-B degradation correlates with the duration of mitosis in GSCs, suggesting that *in vivo*, as inferred from cell culture studies, the SAC also functions in a graded manner. Furthermore, larval GSCs can initiate anaphase with higher levels of non-degraded Cyclin-B as compared to adult cells, suggesting that anaphase entry efficiency declines in aging stem cell populations. In sum, we have developed an assay to monitor degradation of Cyclin-B by APC in the GSCs of an intact, maturing organism. Our results show that mitotic mechanisms are sensitive to the physiological changes of aging, and open the possibility of further studying these mechanisms in a maturing tissue.

P1083**Board Number: B414****3D Print Approaches to Capture Metaphase Cells.**M.R. Castroagudin¹, Y. Zhai², Z. Li², M.G. Marnell², J.S. Glavy³;¹Department of Chemistry, Chemical Biology and Biomedical Engineering, Stevens Institute of Technology, Hoboken, NJ, ²CCBBME, Stevens Institute of Technology, Hoboken, NJ, ³Stevens Inst Technology, Hoboken, NJ

Mitotic spindles form outside the Nuclear Envelope (NE) in higher eukaryotes and the entire NE must disintegrate to facilitate chromosome separation. Discrete units of nuclear pore subcomplexes are released during G2/M phase transition of open mitosis then re-assembled as the cell exits M phase into G1. To track these changes, we rely on the visualization of the NE throughout mitosis using NE rim staining antibodies. HeLa metaphase cells that are synchronized by nocodazole treatment hold condense chromosomes that tend to generate rounded semi-attached cells. Even HeLa Metaphase cells are easily dislodged and require cytopinning to remain attached. In our investigations, we studied several alternative methods to attach these cells and readily visualize the windows of NE breakdown and re-assembly. We found a 3D print alternative to standard cytopin devices to be most effective to retain metaphase cells and visualize the NE throughout the unabridged cell cycle.

P1084**Board Number: B415****Live-Cell Imaging of mES Cells Reveals Diverse Effects of Microtubule Polymerase and Depolymerases on Mitosis.**O. Demir¹, I. Poser¹, A. Hyman², J. Howard³;¹MPI-CBG, Dresden, Germany, ²Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany, ³Molecular Biophysics and Biochemistry, Yale University, New Haven, CT

To capitalize on recent advances in stem cell biology, it is important to understand the physiological properties of stem cells, especially how they divide and multiply. Such studies require live-cell imaging of many cells over many cell cycles. One limitation on imaging embryonic stem (ES) cells is their propensity to form spheres and to undergo apoptosis, making it difficult to image single cells in culture over times comparable to the cell cycle time. Here we describe an imaging system for mouse ES (mES) cells that is suitable for long-term live-cell imaging. The system combines BAC-based gene expression with wide-field deconvolution microscopy for mES cells plated onto the laminin-511-coated-surfaces and kept in CO₂-independent culture conditions. mES cells labeled with TUBB-GFP or H2A-GFP show low rates of apoptosis and aberrant mitosis. We used RNAi in combination with live-cell imaging to analyze the effects of microtubule polymerase and depolymerases on mitosis in ES cells and we performed single and double knockdowns of chTOG, EB1, Kif18A and MCAK to find out their effects in spindle assembly and morphology and as well as mitotic timing and chromosome segregation. Our results show that EB1 and MCAK knock-downs do not influence spindle morphology whereas Kif18A and ch-TOG cause

changes in spindle length without affecting the spindle width and the aspect ratio. The shortening effect of chTOG on spindle length could be partially rescued by EB1 knock-down. In addition, Kif18A strongly affects the timing of mitosis while chTOG and EB1 do not change it. Finally the depletion of each MAP causes chromosome segregation errors in anaphase and they could not be rescued by co-depletions. In conclusion we have generated a pipeline for the use of mouse embryonic stem cells in time-lapse microscopy and we have analyzed the effects of MAPs on spindle and mitotic progression. This method can be easily combined with RNAi technique to further study the cell biology of these unique cells.

P1085

Board Number: B416

Evidence that human tissue culture cells use G2 to prepare for timely anaphase.

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To pass on its genome, a cell must finish replicating DNA before dividing. In the early embryo of *Drosophila* or *Xenopus*, division begins immediately after the completion of DNA replication. In tissue culture, division begins after cells spend hours in G2-phase. What happens during G2 is largely a mystery. We used time-lapse microscopy of MCF10A cells that overexpressed fluorescent PCNA, a reporter of S-phase, and fluorescent histone H2B, a reporter of M-phase, to measure G2 duration. We caused premature mitosis with PD0166285, an inhibitor of Wee1 and Myt1, and found a delay in anaphase onset inversely proportional to G2 duration. Co-treatment with PD0166285 and reversine, an inhibitor of the spindle assembly checkpoint, mostly rescued the delay. Surprisingly, co-treatment with PD0166285 and cycloheximide, an inhibitor of protein synthesis, did not change the anaphase delay, even though treatment with cycloheximide alone largely prevented mitotic entry. We infer that the most important regulation in G2 is not translational, but post-translational or mediated by non-protein-coding RNA. We observed the mitotic spindle by overexpressing fluorescent α -tubulin and found only a slight delay in its formation after shortened G2. Therefore, we speculate that a normal G2 may promote timely attachment of kinetochores to the mitotic spindle.

Cytokinesis 2

P1086

Board Number: B417

Towards a filament-independent model of abscission in *C. elegans*.

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The narrow intercellular bridge connecting the two post-mitotic daughter cells is severed during abscission. This final stage of cytokinesis involves a reorganization of the plasma membrane as well as a coordinated disassembly of the microtubules. It was previously shown that ESCRT-III (endosomal sorting complex required for transport-III) components are involved in abscission in cultured cells, as well as in other membrane scission events, including multivesicular endosome formation and the budding of several retroviruses. In this study, we sought to investigate the potential existence of an ESCRT-independent mechanism for abscission. Taking advantage of the early *C. elegans* embryo, we cryoimmobilized zygotes at various stages of cytokinesis (from furrow ingression up to internalization of the midbody remnant). In contrast to tissue culture cells, our careful time-resolved study did not reveal the accumulation of spiral filaments during the abscission process. In addition, depletion of the ESCRT-I component Tsg101 did not block abscission. To our surprise, we only visualized filamental structures during post-abscission events (membrane shedding). Moreover, we performed drug treatment experiments with Nocodazole, Brefeldin A and Latruncullin A to investigate the roles of vesicle transport and fusion, and the function of actin during abscission. Our data indicate that the drugs affected abscission in distinct ways and at unique stages. By light microscopy, we found that Nocodazole and Brefeldin A inhibit the accumulation of vesicles at the midbody. In addition, Brefeldin A prevents the internalization of the midbody remnant in late stages. This effect was also observed in Latruncullin A-treated embryos. Currently, we are performing electron tomography to study the ultrastructure of the intercellular bridge in such drug-treated embryos to develop a model for abscission in the early *C. elegans* embryo.

P1087

Board Number: B418

Cell polarity contributes to contractile ring assembly and constriction during cytokinesis.

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The tight coordination of cell polarity with division plane specification during cytokinesis is critical for every asymmetric cell division from embryogenesis to the maintenance of adult stem cell niches. The cell polarity machinery was thought to promote division plane specification indirectly, by regulating the position of the mitotic spindle, which then positions the site of contractile ring assembly. Recently, however, studies of *Drosophila* neuroblasts revealed that in the absence of a mitotic spindle, an actomyosin contractile ring still formed at the base of the apical-basal polarity boundary (Cabernard *et al.*, Nature, 2010). A role for the cell polarity machinery in directly regulating cytokinesis was not examined. Here we study the role of cell polarity in directly promoting contractile ring assembly and constriction during cytokinesis in *Caenorhabditis elegans* zygotes. We found that spindle-independent contractile rings also form at the interface of cortical anterior and posterior polarity boundaries, even in the absence of spindle microtubules. In sensitized cytokinesis mutants, disruption of either anterior (PAR-3, PAR-6, CDC-42) or posterior (PAR-2) polarity components weakened actomyosin ring constriction, illustrating a positive role for both anterior and posterior cortical boundaries in cytokinesis. We also found that increasing or decreasing the activity levels of the anterior polarity Rho GTPase CDC-42 led to increased or decreased filamentous actin levels in the contractile ring, respectively. Furthermore, with high-resolution temporal analysis using the Terminator (our newly developed device to rapidly inactivate temperature-sensitive protein function (Davies *et al.* Developmental Cell, 2014)), we found that cell polarity is required for robust constriction of the contractile ring. Specifically, we found a critical window for polarity machinery function early in the cell cycle (during the polarity maintenance phase) that is required for robust contractile ring constriction. Taken together, our data suggest a new role for CDC-42 and the cortical polarity machinery in directly promoting actomyosin contractile ring constriction during cytokinesis in asymmetrically dividing cells.

P1088

Board Number: B419

Characterization of the assembly, regulation and structures of two types of interphase nodes that merge to form cytokinetic nodes in fission yeast.

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Fission yeast assemble a cytokinetic contractile ring from discrete, membrane-bound, multiprotein complexes called nodes. Interactions of myosin-II with actin filaments pull nodes together into a contractile ring. Nodes originate during interphase, but their origins, physical movements and fates were unclear.

We discovered that cytokinesis nodes assemble from two types of interphase nodes composed of different proteins. Type 2 nodes containing protein Blt1p, GTP exchange factor Gef2p, accessory protein Nod1p and kinesin Klp8p exist throughout the cell cycle and emerge from the contractile ring as it disassembles. Type 1 nodes with kinase Cdr1p, kinase Cdr2p and anillin Mid1p follow the separating nuclei and disperse into the cytoplasm during mitosis and reappear in a medial band around each daughter nucleus after mitosis. Quantitative measurements and computer simulations showed that these two types of nodes come together by a diffuse-and-capture mechanism: type 2 nodes diffuse to the equator where they are captured by stationary type 1 nodes.

The Septation Initiation Network (SIN) triggers septation and constriction of the contractile ring in fission yeast. We used conditional mutations to turn the SIN on and off and established that SIN activity is sufficient and necessary to disperse the core type 1 node protein Cdr2p into the cytoplasm. This explains why type 1 nodes assemble only during interphase through early mitosis when SIN activity is low. Activating the SIN during interphase dispersed Cdr2p from type 1 nodes a few minutes after the SIN kinase Cdc7p-GFP accumulated at spindle pole bodies. If the SIN was then turned off in interphase cells, Cdr2p reappeared in nodes in parallel with the decline in SIN activity. Hyperactivating SIN during mitosis dispersed type 1 nodes earlier than normal, and prolonged SIN activation prevented nodes from reforming at the end of mitosis. Conversely, inactivating SIN during mitosis prevented Cdr2p nodes from dispersing into the cytoplasm.

We used live cell FPALM super resolution microscopy to document the structures and motions of interphase nodes at ~ 35 nm resolution. Type 1 and 2 node proteins Cdr2p and Blt1p tagged with mEOS3.2 appeared as discrete structures ~ 50 nm in diameter, similar to Mid1p and consistent with their roles as core structural proteins that scaffold interphase nodes. Imaging cells expressing Blt1p-mEOS3.2 at 200 frames per second revealed nondirectional movements of type 2 nodes in the cell cortex with a diffusion coefficient ~ 400 nm²/s, while type 1 and 2 nodes near the cell equator moved little over 60 s, both requirements for successful simulations of our diffuse-and-capture model.

P1089

Board Number: B420

Tension and constriction of the cytokinetic contractile ring depend on anchoring of ring components to the plasma membrane.

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Cytokinesis involves constriction of a tension-producing actomyosin contractile ring that divides the cell into two. How the ring generates tension remains intensely debated. A sliding filament mechanism where myosin pulls actin filaments was proposed long ago, similar to striated muscle which has sarcomeric organization. However, contractile rings are far more disordered, and it is thought that random actomyosin bundles cannot generate tension. Recently, we presented evidence that tension

originates in anchoring of actin filaments to the membrane (Stachowiak et al., Dev. Cell, 2014) Anchoring allows a disordered arrangement to create tension by providing tension-generating resistance to force. A mathematical model with anchored actin filaments predicted ring tensions close to experimental values ~ 390 pN measured in the same study.

Here we mathematically modeled the constricting ring to explain a recent study of permeabilized fission yeast protoplast that examined ring constriction in controlled environments (Mishra et al, Nature Cell Biol., 2013). The experiments directly tested the role of ring anchoring, as parts of the ring were anchored while others were not: following ATP addition, rings partially detached from the membrane and constricted at a constant rate independent of initial length. Detached portions shortened at ~ 13 $\mu\text{m}/\text{min}$, ~ 50 -fold faster than normal constriction, while anchored portions maintained fixed length.

We extended our molecularly explicit model (Stachowiak et al., Dev. Cell, 2014) to explain these findings and to test the role of anchoring. The model assumes barbed end actin filament anchoring and includes the key components in amounts and with biochemical properties highly constrained by prior experiment. In agreement with the experiments of Mishra et al., simulated detached ring segments had almost zero tension and shortened at a rate somewhat less than twice the load-free velocity of myosin-II, $v_{\text{myo}}^0 \sim 0.14$ $\mu\text{m}/\text{s}$. Our analysis explains why the shortening rate is independent of ring length: detached regions did not contract, but shortened by being reeled in at their end points, each end point contributing v_{myo}^0 . The shortening of a detached portion relies on an adjacent anchored region, into which the detached portion is reeled. Thus, unanchored ring segments have zero tension but nevertheless shorten because they are connected to anchored segments with tension. When all anchoring was abolished, tension and constriction were absent: completely detached simulated rings did not constrict. Simulations reproduced other observations by Mishra et al, including the dependence of constriction on myosin-II but not on actin turnover, and retarded constriction by increased actin crosslinkin

P1090

Board Number: B421

Experimental measurement and simulations of the cytokinetic ring tension in fission yeast.

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Cytokinesis in animals and fungi requires the assembly and constriction of an actomyosin contractile ring at the site of cell division, but the ring tension, the ring's principal mechanical property, has rarely been measured. Consequently, it has not been possible to relate the organization of the ring to its principal

function, and the mechanism of ring constriction has been difficult to establish. We recently developed a method to measure the cytokinetic ring tension in fission yeast for the first time, using yeast protoplasts whose cell walls have been enzymatically digested (Stachowiak *et al.*, *Dev. Cell*, 2014). Using micropipette aspiration to measure the membrane tension, and imaging the geometry of the membrane furrow induced by the tense ring, we deduced the ring tension from a force balance at the furrow. The measured tensions were reproduced by a detailed computer simulation of the constricting ring incorporating key components that have been biochemically and genetically characterized.

The availability of a method to measure cytokinetic ring tension has opened the door to observing the effect of mutations and drug treatments on ring tension that were previously invisible. Even for a mutation with little or no effect on the rate of constriction, the ring tension could be affected since constriction rates are determined by many factors. Here we refined the ring tension measuring technique so that we could track ring tension in real time throughout the full course of constriction, and we measured ring tension in fission yeast mutants, and in wild type cells following drug treatments, where the activity and dynamics of myosin or actin are compromised. We used these results in combination with our mathematical model of the constricting ring to test the basic mechanisms of tension production and constriction.

P1091

Board Number: B422

Dynamic Network Morphology and Tension Buildup in 3D Model of Cytokinetic Ring Assembly.

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During fission yeast cytokinesis, actin filaments nucleated by cortical formin Cdc12 are captured by myosin motors bound to a band of cortical nodes and bundled by crosslinking proteins. The myosin motors exert forces on the actin filaments, resulting in a net pulling of the nodes into a contractile ring, while cross-linking interactions help align actin filaments and nodes into a single bundle. We used these mechanisms in a 3D computational model of contractile ring assembly, with semiflexible actin filaments growing from formins at cortical nodes, capturing of filaments by neighboring nodes, and cross-linking among filaments through attractive interactions. The model was used to predict profiles of actin filament density at the cell cortex, morphologies of condensing node-filament networks, and regimes of cortical tension by varying the node pulling force and strength of crosslinking among actin filaments. Results show that cross-linking interactions can lead to confinement of actin filaments at the simulated cortical boundary. We show that the ring formation region in parameter space lies close to regions leading to clumps, meshworks or double rings, and stars/cables. Since boundaries between regions are not sharp, transient structures that resemble clumps, stars and meshworks can appear in the process of ring assembly. These results are consistent with prior experiments with mutations in actin filament turnover regulators, myosin motor activity and changes in the concentration of cross linkers that alter the morphology of the condensing network. Transient star shapes appear in some simulations, which

offer an explanation for star structures observed in prior experimental images. Finally, we quantify tension along actin filaments and forces on nodes during ring assembly and show that the mechanisms describing ring assembly can also drive ring constriction once the ring is formed.

P1092

Board Number: B423

Back-to-back mechanisms drive actomyosin ring contraction during *Drosophila* cellularization.

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While Myosin-2 motor activity is required for actomyosin ring contraction during cytokinesis in some cell types, recent findings suggest that motor activity is dispensable in other cell types, including mammalian cultured cells and budding yeast. One simple possibility is that different cell types use different mechanisms of actomyosin contraction during cytokinesis. Another possibility is that Myosin-2 dependent and independent mechanisms act in the same cell type, but make different contributions to contraction. In this work, we address the contribution of Myosin-2 motor activity during the cytokinetic event of cellularization in early *Drosophila* embryos.

During cellularization, actomyosin rings contract to build the bottom of newly forming epithelial cells. By quantitative live cell imaging, we find that ring contraction proceeds in two morphologically and kinetically distinct phases. In Phase 1, hexagonal rings become circular, and the contraction rate is slow ($0.17 \pm 0.04 \mu\text{m}/\text{min}$, mean \pm S.E.M.). In Phase 2, rings get smaller, and ring contraction is fast ($0.66 \pm 0.03 \mu\text{m}/\text{min}$, mean \pm S.E.M.). F-actin and Myosin-2 levels increase or stay constant, respectively, during Phase 1, but both decrease during Phase 2, suggesting that the Phases are mechanistically distinct. Indeed, using genetic mutants and drug strategies to manipulate Myosin-2 motor activity and F-actin polymerization dynamics, we find that contraction during Phase 1 depends on Myosin-2 motor activity, while Phase 2 does not. Instead, Phase 2 contraction depends on F-actin depolymerization.

Taken together, our work shows that two back-to-back mechanisms drive distinct phases of actomyosin ring contraction during cellularization. Our data supports a model whereby Myosin-2 dependent and independent mechanisms conspire to drive ring contraction, within the same cell type, and even during the same cytokinetic event. Cellularization now provides a unique opportunity to compare distinct contraction mechanisms in the same cell type, and to understand what molecular components control the switch between Myosin-2 dependent and independent mechanisms.

P1093**Board Number: B424****Generating an Asymmetric Furrow: The Roles of Anillin and F-actin Alignment.**K. Rehai¹, J. Dorn², L. Zhang², B. Lacroix³, A.S. Maddox⁴;¹Genetics and Molecular Biology, University of North Carolina, Chapel Hill, NC, ²Pathology and Cell Biology, University of Montreal, Montreal, QC, ³Institut Jacques Monod, Paris, France, ⁴Department of Biology, University of North Carolina, Chapel Hill, NC

Cytokinesis, the physical division of a cell, is driven by the closure of a ring enriched for the actomyosin cytoskeleton. Despite the necessity of proper ring formation and function, the organization of ring components is still poorly understood.

In the *C. elegans* zygote the cytokinetic ring closes asymmetrically, a conserved feature of cytokinesis seen throughout Metazoa. In other systems, the asymmetry of ring closure can be explained by a pre-existing asymmetry such as an eccentric spindle, apical junctions, or substrate adhesions. However, the *C. elegans* zygote is a mechanically isolated cell, indicating that cell intrinsic processes can generate furrow asymmetry and that understanding asymmetry will reveal insight into general principles of cytokinesis. We propose that cell-intrinsic asymmetry is generated by differential organization of ring components on the faster versus slower ingressing side of the ring. This asymmetry of cytokinetic ring closure is dependent on the *C. elegans* protein ANI-1, a homologue of Anillin, which scaffolds structural and regulatory components of the ring. Importantly, the predicted actin binding domain of ANI-1 is sufficient to bundle F-actin *in vitro*. To assess the relationship between actin organization and asymmetry we developed a novel image analysis tool and found that F-actin is progressively circumferentially aligned at the cell equator just as cytokinesis initiates, and that this alignment is dependent on ANI-1.

These results demonstrate a positive correlation between F-actin alignment and asymmetry, supporting a model in which localized F-actin alignment activates a positive feedback loop causing further localized alignment, and ultimately generating asymmetry within the cytokinetic ring. To further test this model we generated a *C. elegans* strain expressing the isolated ANI-1 actin bundling domain tagged with GFP as well as a strain expressing ANI-1 lacking its actin bundling domain tagged with GFP. ANI-1 Δ actin-bundling-domain::GFP localizes to the cytokinetic ring, while the isolated ANI-1 actin-bundling-domain does not. In the presence of endogenous full length ANI-1, neither truncation has a clear dominant effect on cytokinesis. In ongoing work, we are testing the ability of these truncations to rescue depletion of endogenous ANI-1 by RNAi.

P1094**Board Number: B425****Cleavage-furrow ingression and F-actin dynamics in the green alga******Chlamydomonas reinhardtii****M. Onishi¹, L. Mackinder², M.C. Jonikas², J. Pringle¹;¹Genetics, Stanford Univ Med Ctr, Stanford, CA, ²Plant Biology, Carnegie Institution for Science, Stanford, CA

Cytokinesis in animals and fungi is achieved by ingression of the cleavage furrow, which involves a ring containing F-actin, type-II myosin, and functionally related proteins. This “contractile actomyosin ring” has long been thought to provide the force for ingression by a sliding interaction between actin and myosin filaments. However, the phylogenetic distribution of type-II myosin is confined to fungi, animals, and slime molds, whereas most other eukaryotes also divide by furrowing. To address this paradox, we are investigating the mechanisms of cleavage-furrow ingression in one such eukaryote, the green alga *Chlamydomonas reinhardtii*. Not only does *Chlamydomonas* have no type-II myosin, but the very existence of F-actin in vegetative cells has also been controversial. Although it has been reported that actin is enriched at the cleavage furrow, it was not labeled by phallotoxins, and a null mutation in the single conventional actin gene (*IDA5*) does not cause a significant growth defect. However, we have now demonstrated the existence of F-actin by developing a novel method to overexpress a Lifeact-Venus probe that should label only F-actin. In interphase cells, Lifeact-Venus localized to the mid-portion of the cell, forming a cage-like structure around the nucleus and to the flagellar-basal region as cortical dots; in dividing cells, it was found in the cleavage furrows. These signals were sensitive to the actin-depolymerizing latrunculins, confirming that they represent F-actin. In addition, the type-VIII myosin (the other two *Chlamydomonas* myosins are of type XI) has been successfully tagged with Venus and showed localization to the mid-cell cage and the flagellar-basal cortical dots, but not to the cleavage furrow. Latrunculin-treated cells continued to grow at a nearly normal rate with no apparent defect in cell division. However, these cells, like the *ida5* null mutant, had upregulated expression of a gene (*NAP*) encoding an unconventional actin (~65% identical to conventional actins), and the Nap filaments were resistant to the drug. These results show that *Chlamydomonas* has a surprising and robust mechanism to sense and react to the acute loss of F-actin structures, suggesting a requirement of F-actin for some important biological processes in this organism. However, the absence of a type-II myosin and the failure of the one myosin examined to date to localize to the cleavage furrow suggest that formation of a contractile actomyosin ring is not such a process. We will report on our ongoing genetic and molecular investigations of the F-actin-sensing mechanism and the mechanisms of cytokinesis in *Chlamydomonas*; these studies should illuminate both the roles of F-actin and the ancestral mechanisms of cleavage-furrow formation.

P1095**Board Number: B426****A chromatin microtubule-independent pathway driven by Ran-GTP helps define the division plane during cytokinesis.**T. Akhshi¹, A.J. Piekny¹;¹Department of Biology, Concordia University, Montreal, QC

Cytokinesis describes the physical separation of a mother cell into two daughter cells and is driven by the constriction of an actomyosin contractile ring. Formation and ingression of the contractile ring is regulated by the mitotic spindle; the central spindle recruits Ect2, a GEF that activates RhoA, to mediate ring assembly in the equatorial plane, while the astral microtubules restrict the localization of contractile proteins at polar cortices via a molecular mechanism that involves anillin. However, contractile proteins equatorially accumulate in the absence of microtubules, suggesting that redundant pathways regulate their localization. Here, using human Hela cells, we have provided evidence that a chromatin-based pathway involving Ran-GTP restricts the localization of contractile proteins. We found that there is a strong correlation between the boundary of equatorially accumulated anillin and chromatin position. We calculated the minimum distance from which cue(s) associated with chromatin could function in cells with intact spindles, and in cells lacking astral and/or central spindle microtubules and altered geometry. Furthermore, by targeting active Ran to the furrow or inactivating Ran in bipolar cells, we found that Ran-GTP negatively regulates the localization of contractile proteins. Our studies shed light on a chromatin-based pathway that likely functions to couple the division plane with the segregation of sister chromatids, which becomes essential when the mitotic spindle is perturbed or shifts too close to one pole.

P1096**Board Number: B427****Control and function of microtubule overlaps in the bipolar phragmoplast network.**J. de Keijzer¹, J. Verweij¹, T. Miki², G. Goshima², M. Janson¹;¹Laboratory of Cell Biology, Wageningen University, Wageningen, Netherlands, ²Division of Biological Science, Nagoya University, Nagoya, Japan

Constriction of the plasma membrane during cell division in animal cells is regulated by short regions of overlapping microtubules at the centre of the cell division apparatus. Similar regions of limited antiparallel microtubule overlap are found in the phragmoplast of plants, a bipolar microtubule network that constructs a cell plate to separate two daughter cells. We used the genetically tractable moss *Physcomitrella patens* to gain insight into the function and regulation of these overlaps. We found that overlaps act as recruitment sites for vesicles containing cell wall material. The short length of overlaps may thus be a prerequisite to precisely pattern the forming cell plate. To understand the control of

overlap length we investigated the balance between local rates of microtubule growth and microtubule sliding. The first activity extends overlaps whereas the second decreases overlap length and drives microtubule flux. By tracking growing microtubule ends and regions of photo-activated microtubule filaments we show that the bulk microtubule growth velocity is several times faster than the rate of relative sliding, implying that microtubule growth must be locally down regulated within overlaps. We therefore functionally analysed kinesin-4 proteins. In agreement with an established role in inhibiting microtubule growth, deletion of two kinesin-4 genes caused oscillations in overlap length. Moreover, the initial recruitment of vesicles to overlaps was more diffuse, cell plate construction was delayed, and completed cell plates were thicker and lacked structural integrity. Our results thus demonstrate the patterning of the cell plate by the phragmoplast involves kinesin-4 mediated length control of microtubule overlaps.

P1097

Board Number: B428

The kinesin-3 motor KIF14 exhibits distinct biochemical properties and structural features.

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The mitotic kinesin-3 motor protein KIF14 is essential for cytokinesis during cell division. KIF14 has been found overexpressed in multiple cancers and its overexpression has been linked to poor prognosis in cancer patients. Therefore, KIF14 is sometimes referred to as the oncogenic kinesin. We characterized the motor domain of murine KIF14 biochemically and solved its crystal structure in the ADP-bound state. Our data show that KIF14 motor binds tightly to microtubules and does not display typical nucleotide-dependent changes in this affinity. It has robust ATPase activity but very slow motility. Interestingly, our ADP•KIF14 motor domain crystal structure reveals a drastically opened nucleotide-binding pocket, as if ready to exchange its bound ADP for Mg•ATP. In this state, the central β -sheet is twisted $\sim 10^\circ$ beyond the maximal amount observed in other kinesins. This configuration has only been previously observed in the nucleotide-free states of myosins-known as the "rigor-like" state. Fitting of this atomic model to electron density maps from cryo-electron microscopy indicates a distinct binding configuration of the motor domain to microtubules. We postulate that these properties of KIF14 are well suited for stabilizing microtubules at the midbody during cytokinesis.

P1098**Board Number: B429****Cdk1-dependent phosphorylation of leukemia-associated RhoGEF (LARG) during mitosis.**M.C. Helms¹, E. Grabocka¹, M.K. Martz¹, N. Suzuki², P.B. Wedegaertner¹;¹Department of Biochemistry and Molecular Biology, Thomas Jefferson University, Philadelphia, PA,²Department of Signal Dynamics, University of Tokyo, Meguro, Japan

Rho GTPases are crucial players in the regulation of actin cytoskeleton-dependent processes, including mitosis. Our laboratory has discovered a novel role and localization of the leukemia-associated Rho guanine-nucleotide exchange factor (LARG), a regulator of G protein signaling (RGS)-RhoGEF, during mitosis. Rho and LARG have been implicated in aberrant mitosis and embryonic development, as well as diseases such as hypertension and cancer, thus highlighting the importance for further research. The purpose of this study is to determine the molecular mechanism and role of mitotic-dependent phosphorylation of LARG. Our experiments are conducted in vitro, in HeLa cells, a human uterine cancer cell line, and human embryonic kidney (HEK293) cells, and experiments are conducted with cellular, molecular, and biochemical approaches. Using mitotic-shift assays and kinase inhibitors, we report that LARG undergoes a mitotic-dependent and Cyclin-dependent kinase 1 (Cdk1) inhibitor-sensitive phosphorylation. Cell synchronization followed by Western blot also identified the temporal feasibility of Cdk1 as a potential kinase. Furthermore, using in vitro kinase assays, we show that LARG can be directly phosphorylated by Cdk1. Using both N- and C-terminal deletion and multi-site phosphorylation mutants, we demonstrate that the mitotic-dependent shift of LARG relies on phosphorylation occurring in both termini. Using custom phosphospecific antibodies, we confirm that two sites are phosphorylated during mitosis and also in a Cdk1-dependent manner. Luciferase reporter assays for RhoA activity are being used to assess differences in RhoA activation depending on phosphorylation status of LARG. We conclude that Cdk1 phosphorylates LARG during mitosis, and phosphorylation of LARG may play a role in LARG activity and function. Since LARG has diverse signaling functions beyond mitosis such as in the heart and in embryonic development, it is important to understand the regulation of the protein.

P1099**Board Number: B430****Large tumor suppressors 1 and 2 regulate the centrosomal activation of LIM-kinase 1 by phosphorylating a motor protein CHO1.**A. Okamoto¹, N. Yabuta¹, S. Mukai¹, K. Torigata¹, H. Nojima¹;¹Osaka Univ, Suita, Osaka, Japan

Large tumor suppressor 1 and 2 (Lats1/2) are core Ser/Thr kinases of the Hippo signaling pathway that controls organ size by modulating cell proliferation and cell death. Lats1/2 also play important roles in mitosis by regulating centrosomal integrity, chromosome segregation and cytokinesis. As components of

the centralspindlin complex, the kinesin-like motor protein CHO1 and its splicing variant MKLP1 regulate the formation of the contractile ring and completion of cytokinesis by colocalizing with chromosome passenger proteins and GTPases. However, the regulatory mechanisms of CHO1 remain unclear. Here, we show that Lats1/2 regulate cytokinesis by directly phosphorylating Ser716 in the F-actin binding region of CHO1, which is absent in MKLP1. We found that CHO1-pS716 localizes to the centrosomes and midbody during mitosis and cytokinesis, respectively. Furthermore, we also found that LIM-kinase 1 (LIMK1), a modulator of actin dynamics, interacts with the Lats1/2-mediated S716-phosphorylated form of CHO1. Indirect immunofluorescence and immunoblotting analyses revealed that overexpression of phosphorylated and non-phosphorylated mutants of CHO1 altered the mitotic localization and activation of LIMK1 at the centrosomes in HeLa cells, leading to the cytokinesis failure through excessive phosphorylation of Cofilin, an actin depolymerization factor, and mislocalization of Ect2, a Rho guanine nucleotide exchange factor. These results suggest that Lats1/2 stringently regulate cytokinesis by phosphorylating CHO1, leading to interaction with and subsequent activation of LIMK1 on centrosomes during early mitosis. Therefore, we propose that the Lats1/2-CHO1-LIMK1 axis is a pivotal signaling pathway that links centrosome integrity and the fidelity of cytokinesis to prevent polyploidization.

P1100

Board Number: B431

Cdc42 Signaling Pathway Drives Onset of Actomyosin Ring Constriction During Cytokinesis.

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Cytokinesis, the last stage in cell division is a complex process involving multiple steps. During cytokinesis in fission yeast, first the precursor nodes localize to the cell middle which then recruit proteins that form the actomyosin ring. Next, the ring undergoes maturation followed by constriction (1). A leading question in the field is what triggers actin ring constriction. Here we show for the first time a potential role for the conserved GTPase Cdc42 in promoting the onset of actomyosin ring constriction during cytokinesis. Our results show that Cdc42 activation occurs during ring maturation and persists through ring constriction. Cdc42 is activated by Guanine nucleotide Exchange Factor (GEF), which promotes GTP binding (2). Loss of the Cdc42 GEF Gef1 does not show Cdc42 activation during ring maturation and constriction. Further, upon loss of gef1 the ring persists in the maturation phase longer and onset of ring constriction is delayed. Cells lacking gef1 also display fewer non-medial actin cables that are known to incorporate in to the ring. Non-medial actin cables are dependent in the actin polymerizing formin Cdc12. Here we show that loss of gef1 mutants display increased mobility of Cdc12 within the ring as determined by FRAP analysis. Taken together these observations suggest that Gef1 activates Cdc42 during actin ring maturation to promote stabilization of Cdc12. Stabilized Cdc12 possibly leads to increased actin polymerization and non-medial cable formation. Further we propose that incorporation of non-medial actin cables in to the ring during maturation is required for the onset of actin ring constriction. Current experiments are focused on studying the above hypothesis.

Keywords: Cytokinesis, Cell Division, Acto-myosin ring, actin, formin, Cdc42, Cdc12, Fission Yeast, Gef1

Oncogenes and Tumor Suppressors 2

P1101

Board Number: B433

The identification of novel putative partners of USP22, oncoprotein and potential chemotherapeutic target.

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Cancer stem cells play a fundamental role in tumor development and metastasis in many forms of cancer, including chronic myelogenous leukemia, acute lymphoblastic leukemia, and breast cancer. The transformation of normal stem cells to cancer stem cells may be mediated through epigenetic mechanisms, some of which have been reported to involve Polycomb group proteins. Recently, an 11-gene signature, including Polycomb group genes, for aggressive cancers was identified; this signature includes USP22, which codes for a histone ubiquitin hydrolase. USP22 acts as part of the human SAGA complex in the nucleus to activate transcription of Myc via histone deubiquitination. However, USP22's role in the cytoplasm, beyond its association with SIRT1, is poorly characterized. In order to better understand USP22's cytoplasmic activity, we conducted tandem mass spectrometry of FLAG-tagged USP22 immunoprecipitates, identifying over twenty potential novel partners of USP22 in the cytoplasm. Subsequent purification of the USP22 core enzymatic complex, consisting of USP22, ATXN7L3, ATXN7, and ENY2, has allowed for screening of a small molecule library for inhibitors of USP22's enzymatic activity. Such inhibitory compounds represent potential chemotherapeutic agents and will harness current understanding of USP22's role in aggressive cancers.

P1102

Board Number: B434

The role of Par1b during *Helicobacter pylori* infection.

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Gastric carcinoma, the second leading cause of cancer-related deaths in the world, has been linked to infection with *Helicobacter pylori*, a bacterium that colonizes the gastric mucosa. *H. pylori* strains that express a gene called cytotoxin-associated gene A (CagA), present a much higher cancer risk for their host than strains that lack CagA and it has been shown to function as an oncogene in mice. CagA induces loss of cell polarity, increased cell migration and apoptosis of gastric epithelial cells and promotes aberrant growth signals by activating the Ras-MAP-cascade. Recently, studies from our group and others

have identified the serine/threonine kinase Par1 as a CagA-target. Here we utilized *H. pylori*-infected polarized human primary gastric epithelial cells (HGECs) and a human adenocarcinoma cell line (AGS) to demonstrate that Par1b inhibition contributes to a CagA-dependent activation of DNA double strand breaks and the initiation of DNA double strand repair (DDR) upon acute *H. pylori* infection as well as to the activation of the Jnk kinase. Ongoing work is aimed at identifying the Par1b substrates involved.

P1103

Board Number: B435

LisH2 is required for β -catenin nuclear localization and associated with colon cancer.

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Canonical Wnt signaling pivotally regulates cell fate and proliferation during embryonic development and tumorigenesis. Nuclear accumulation of β -catenin is the central event of canonical Wnt signaling cascade. However, the molecular mechanisms that control β -catenin nuclear localization remain poorly understood. Here, we identify LisH2 as a critical downstream component of Wnt/ β -catenin signaling, which is required for Wnt induced β -catenin nuclear localization. Wnt increases LisH2 protein level and promotes LisH2 nuclear accumulation through the inactivation of the Axin complex. Either depletion of LisH2 or LisH2 mutation that disrupts LisH2- β -catenin interaction abolishes nuclear localization of β -catenin in response to Wnt signaling. Furthermore, LisH2-mediated β -catenin nuclear accumulation is essential for proper dorsoventral patterning of zebrafish embryos and colonic tumor progression. Thus, our data suggest a previously undescribed mechanism whereby LisH2 facilitates β -catenin nuclear localization in canonical Wnt signaling.

P1104**Board Number: B436****Wnt/ β -catenin signaling enhances RUNX1 expression in leukemia cells and hematopoietic progenitors.**G.D. Ugarte¹, M.A. Medina¹, M.F. Vargas¹, A.A. Elorza¹, A. Loyola², G.V. De Ferrari¹;¹Center for Biomedical Research, Universidad Andres Bello, Santiago, Chile, ²Fundación Ciencia Vida, Santiago, Chile

Canonical Wnt/ β -catenin signaling has a crucial role in proliferation and differentiation of haematopoietic lineages and its deregulation is thought to be involved in leukaemia. Similarly, the transcription factor RUNX1 is required for definitive haematopoiesis and is frequently rearranged in various types of leukaemia. Here we report that RUNX1 expression is readily induced in leukemia cell lines HL-60 and Jurkat and CD34+ primary human hematopoietic progenitors in response to induction with a purified Wnt3a protein or pharmacological treatments with lithium chloride. Serial deletion of a 1.7 Kbp of the P1-distal promoter fragment allowed us to identify a minimal β -catenin responsive region, which showed maximal response in gene-reporter assays, that includes a novel T-cell factor/lymphoid enhancer factor (TCF/LEF)-response element (TBE Site II), located upstream of the canonical RUNX1 transcription start site. This observation was further confirmed by site directed mutagenesis and chromatin immunoprecipitation (ChIP) assays. Our results link the transcription of the RUNX1 gene to Wnt/ β -catenin signaling and may open a novel window to understand the development or the deregulation of the hematopoietic process.

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P1105**Board Number: B437****CDO, an Hh-coreceptor, mediates lung cancer cell proliferation and tumorigenicity through Hedgehog signaling.**J. Bae¹, Y. Leem¹, K. Baek¹, J. Kang²;¹School of Medicine, Sungkyunkwan University, Suwon, Korea, ²Sungkyunkwan Univ Sch Med, Suwon, Kyunggi-do, Korea

Hedgehog (Hh) signaling plays essential roles in various developmental processes, and its aberrant regulation results in genetic disorders or malignancies in various tissues. Hyperactivation of Hh signaling is associated with lung cancer development, and there have been extensive efforts to investigate how to control Hh signaling pathway and regulate cancer cell proliferation. In this study we investigated a role of CDO, an Hh co-receptor, in non-small cell lung cancer (NSCLC). Inhibition of Hh signaling by SANT-1 or siCDO in lung cancer cells reduced proliferation and tumorigenicity, along with the decrease in the expression of the Hh components. Histological analysis with NSCLC mouse tissue demonstrated that CDO was expressed in advanced grade of the cancer, and precisely co-localized with GLI1. These data

suggest that CDO, most likely via Hh signaling activation, is required for proliferation and survival of lung cancer cells via Hh signaling.

P1106

Board Number: B438

Regulation of Na,K-ATPase by Sonic hedgehog signaling in cerebellar granule precursor cells.

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Na,K-ATPase is a membrane protein that maintains intracellular ion homeostasis, and is responsible for generating ion gradients across cell membranes. It consists of a catalytic alpha subunit, and a beta subunit that pumps sodium ions out and potassium ions into the cell using ATP. Studies from our laboratory and others have established additional roles for Na,K-ATPase as a signaling scaffold and a cell adhesion molecule. Some of the signaling pathways modulated by Na,K-ATPase have been linked to cancer progression including cell growth, cell adhesion, and cell motility. Changes in Na,K-ATPase function and expression have been reported in various cancers and may occur at very early stages of tumorigenesis, suggesting that altered Na,K-ATPase subunit expression and function may contribute to tumor development and progression.

The sonic hedgehog pathway (Shh) is an important signaling pathway involved in cerebellar development. Shh is secreted from the Purkinje cells, and acts as a mitogen for cerebellar granule precursor (CGP) cells located in the external granule layer of the cerebellum. Mutations leading to hyperactive Shh signaling have been associated with medulloblastoma, which is a common form of pediatric brain cancer thought to originate from improper migration and differentiation of CGP cells. The molecular targets contributing to Shh-mediated proliferation and differentiation of these cells are still poorly understood. We now found reduced Na,K-ATPase beta1-subunit expression in tumors of a transgenic medulloblastoma mouse model with aberrant activation of Shh signaling and activation of Shh signaling prevented upregulation of the beta1 subunit in primary CGP cultures from wild-type cerebella. In addition, shRNA-mediated beta1 knockdown increased cell proliferation, and subcutaneous xenografts of beta1 knockdown cells grew faster than control xenografts. We further show that the polycomb transcription factor Bmi1 that is induced upon activation of Shh signaling may suppress beta1-subunit expression, and we suggest that Bmi1-mediated repression of Na,K-ATPase beta1 subunit may contribute to tumor progression in Shh-mediated medulloblastoma.

P1107**Board Number: B439****CD44 intracytoplasmic domain (CD44-ICD)-mediated signaling pathway in breast cancer cells.**D.A. Grinnage¹, N. Gadde², K.E. Miletti-Gonzalez¹;¹Department of Biological Sciences, Delaware State University, Dover, DE, ²Department of Health Sciences, University Of Delaware, Newark, DE

The expression of CD44 in breast cancer cells has been associated with increased cell aggressiveness, which involves changes in cell migration, invasion, adhesion and multi-drug resistance. We have shown that CD44 is able to modulate the cancer cell phenotype in part by regulating the expression of key cancer-related genes through its intracytoplasmic domain (CD44-ICD), a product of the cleavage of its C-terminus by the gamma secretase complex. The CD44-ICD is commonly translocated to the nucleus where it can interact with its response element (CIRE) and/or with the transcription factor Runx2/AML3. Based on these previous findings, we hypothesize that CD44 has the ability to regulate multiple genes and potentially interact with additional transcription factors. Using transcription factor-specific profiling plate arrays to analyze nuclear extracts, we compared the expression of 96 transcription factors in MCF-7 (low invasive negative CD44) and MCF-7/CD44 (highly-invasive CD44 positive stable transfectant) cells. Preliminary data from this analysis indicates that the expression of CD44S correlates with the regulation of numerous genes encoding transcription factors relevant in cancer. We also analyzed the potential protein-protein interaction with other transcription factors using the same plate array methodology. We found that several transcription factors do interact with the CD44-ICD including some that its gene expression is regulated by CD44. Some of the TF/CD44-ICD protein-protein interactions will be validated by chromatin immunoprecipitation (ChIP) assays. Identification and validation of these interactions will provide mechanistic information of the interaction as well as the identity of the genes regulated by the CD44-ICD mediated signaling pathway.

P1108**Board Number: B440****The conserved aromatic loop of human RECQ1 helicase is required for catalytic strand separation vital for an intact replication stress response.**T. Banerjee¹, J.A. Sommers¹, J. Huang¹, M.M. Seidman¹, R.M. Brosh¹;¹National Institute on Aging (NIA/NIH, Baltimore, MD)

RECQ1, a RecQ helicase important for genomic stability, is highly expressed in a spectrum of cancers; however, its precise roles are poorly understood. We used genetic and biochemical assays to dissect RECQ1's role in DNA replication and chromosomal integrity. Two highly conserved residues in the aromatic loop of the RECQ1 helicase core domain were replaced by site-directed mutagenesis (F231A, W227A) and the mutant recombinant proteins purified. Both mutants bound DNA similar to RECQ1-WT;

however, unwinding of forked duplex DNA substrates was severely compromised and branch-migration of mobile D-loops or Holliday Junctions was completely abolished. In contrast, RECQ1-W227A or -F231A were modestly compromised in ATP hydrolysis and retained their ability to bind ATP, oligomerize, and catalyze efficient strand annealing. Therefore, the RECQ1 aromatic loop is critical for efficient coupling of ATPase to DNA unwinding or branch-migration. Genetic rescue experiments in RECQ1-depleted HeLa cells expressing either RECQ1 aromatic loop mutant revealed a markedly reduced replication rate and consequent random firing of dormant replication origins as demonstrated by single molecule DNA fiber assays. These cells displayed significantly elevated spontaneous double-strand breaks and sensitivity to the DNA damaging agents camptothecin (CPT) or H₂O₂. Moreover, expression of either mutant exerted a dominant negative effect on replication rate and sensitivity to exogenously induced DNA damage. CPT-treated RECQ1-depleted cells expressing either RECQ1 aromatic loop mutant revealed a defect in RPA foci formation. The damage induced RPA foci were restored in RECQ1-depleted cells overexpressing RPA, suggesting RECQ1 deficiency leads to random activation of dormant origins which recruit RPA to sites of nascent DNA synthesis, thereby exhausting the pool of RPA available to localize at DNA damage sites, resulting in elevated double strand breaks. MRE11 foci formation was also defective in CPT-treated RECQ1-depleted cells, suggesting a problem in much earlier events of DNA repair upstream of RPA recruitment. In addition, RECQ1-depleted cells expressing either catalytically defective aromatic loop mutant accumulated more CPT-induced DNA damage and bypassed the replication checkpoint, allowing faster growth potentially pushing the cells towards tumorigenesis. Taken together, our results demonstrate that catalytic inactivation of RECQ1 helicase disrupted normal replication and the DNA damage response. Furthermore, RECQ1 is critical to maintain normal replication rate and suppress dormant origin activation, thereby making RPA available to recruit to both endogenous and exogenous induced DNA damage sites. Our findings reveal a potential tumor suppressor role of RECQ1.

P1109

Board Number: B441

Coordinated Regulation of Exo1 in DNA End Resection.

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The cellular response to DNA double-strand break (DSB) damage is essential for the maintenance of genome stability and the prevention of an array of systemic human diseases such as cancer, premature ageing, neurological and developmental disorders. The DNA end resection process dictates the DSB damage response through activating DNA repair by homologous recombination and the ATR checkpoint response. Although a myriad of protein factors have been identified in the DNA end resection pathway, it is still not clear how the extent of end resection and its termination are regulated. Our studies in human cells and *Xenopus* egg extracts indicate that the major resection nuclease Exo1 is regulated both positively and negatively by multiple protein-protein interactions to ensure a proper level of DNA

resection. We find that poly(ADP-ribose) (PAR) binding to the N-terminal catalytic domain of Exo1 initiates Exo1 damage recruitment, while PCNA interaction with the C-terminal domain of Exo1 facilitates Exo1 retention at damaged chromatin and enhances its resection processivity. In contrast, association of 14-3-3 proteins with the central region of Exo1 negatively regulates the recruitment and DNA end resection of Exo1 by inhibiting Exo1 interaction with PAR and PCNA. Furthermore, our work suggests a feedback mechanism for resection termination: DNA end resection leads to activation of ATR and its downstream kinase Chk1, which, in turn, phosphorylates Exo1, creating high-affinity binding site(s) for 14-3-3 proteins. Binding of 14-3-3s then inhibits Exo1 damage association, leading to termination of DNA resection. Collectively, our results provide important mechanistic insights into the coordinated regulation of Exo1 and the DNA end resection process.

P1110

Board Number: B442

CONSTITUTIONAL PREDISPOSITION TO DNA DOUBLE STRAND BREAKS DEFINES A NEW CLASS OF FAMILIAL COLON CANCER.

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Colorectal cancer (CRC) is the second most common fatal cancer in the U.S. with ~ 30% familial cases. Genetic predispositions have only been defined for 25% of familial CRC (FCRC). For the undefined group (uFCRC), current screening methods lack a molecular diagnosis and understanding of the natural history of disease, resulting in under- and over-screening. The known deficits cause DNA repair abnormalities that result in base changes. We hypothesized that uFCRC would originate from a distinct form of genetic instability: defective suppression of DNA double-strand breaks (DSBs). We assayed primary T-lymphocytes from uFCRC patients for the presence of the DSB marker γ H2AX and compared them to age- and sex-matched controls. Elevated γ H2AX levels were observed in 12/21 patients vs. 1/10 controls (P = 0.008). Exome sequencing in the patients (N = 24) identified typically 1 to 3 rare, high quality gene variants that are predicted to damage protein function, are involved in pathways that suppress and/or repair DSBs to maintain normal cell cycle progression and have not been previously implicated in CRC. Variants were enriched in nucleotide excision repair (P = 0.0015), Fanconi's anemia (P = 0.015), and DNA polymerase (P = 0.03) proteins. Patients with polyposis had more variants (P = 0.01). These variants were enriched for genes related to mitosis (P = 0.005) and included novel cell proliferation/signaling

related variants in Adenomatous Polyposis Coli, BMP7, and CDKN3, suggesting features of potential importance for tumor initiation. In all, eight variants map at or near the 26 loci linked previously to CRC risk. Next, we studied one patient in detail. Metaphase spreads exhibited greater sporadic chromosomal gains (8%) than in controls (1.4 %). Flow cytometry showed a high G2/M fraction in the patient than controls. This patient harbored candidate disease-causing variants in 3 DNA DSB repair genes: ERCC6, WRN, and FAAP100. Both the WRN and ERCC6 variant proteins were dysfunctional in biochemical tests. We next established EBV transformed cell lines from this patient and controls. The patient-derived lines revealed, at baseline and in response to several DNA damaging agents, elevated levels of γ H2AX and larger nuclear comets, a second indicator of DSBs. This phenotype could be rescued by expression of wild type WRN or ERCC6. In contrast, expression of the WRN or ERCC6 variants failed to rescue the phenotype. Further, the phenotype could be induced by knockdown of WRN or ERCC6 in CRC cells. We propose that defects in a number of genes that suppress DSBs cause constitutional genomic instability that presents as uFCRC. Identifying novel genetic subsets of FCRC, would clarify the natural histories of disease in these groups and improve screening/diagnosis.

P1111

Board Number: B443

Exploring the Functional Relationship Between CDK6 and the EYA Proteins.

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Cyclin-dependent kinase 6 is a member of a family of kinases that regulate DNA replication. CDK6 phosphorylates pRB, which releases E2F, which in turn goes on to activate the transcription of genes necessary for cell proliferation (Gossel, 2006). It has recently been proven that CDK6 also binds to and promotes the degradation of EYA2 protein, which may in turn allow the cell to proceed into the S phase of the cell cycle (Gossel, 2014). The purpose of our work was to see if CDK6 also interacted with family member protein, EYA3. This is important as the overexpression of EYA proteins has been known to lead to many different types of cancer. To begin, EYA3 protein was produced and detected, as shown in figure 4. Then, an IP was performed using cell lysates that overexpressed both EYA3 and CDK6. The CDK6-conjugated antibody was collected from the lysates and the bound proteins were run on a western blot. EYA3 was also detected on this blot as shown in figure 5. The results suggest that CDK6 does indeed bind EYA3 protein.

P1112**Board Number: B444****Exploring the Relationship between CDK6 and EYA Family Member Proteins.**A. Guerrero¹, J. Blair¹, M. Gossel¹;¹Department of Biology, Connecticut College, New London, CT

Studies have shown that cyclin-dependent kinase 6 plays an important role in cell proliferation by phosphorylating the protein pRB making it inactive. Inactivation of pRB releases it from the E2F protein. E2F can then go on to initiate transcription of genes needed for DNA replication in the S-phase of the cell cycle. (Gossel, 2006) CDK6 has been linked to cancer because overexpression of this protein blocks differentiation and promotes cell division. A recent discovery reveals that CDK6 is not only involved in cell proliferation and differentiation, but also binding to and mediating EYA2 degradation. (Gossel, 2014). The EYA proteins are a family of regulatory proteins that promote cell proliferation and are also essential for organogenesis. Overexpression of these proteins is associated with breast, ovarian, cervical, hematopoietic cancers, and lung adenocarcinoma. Further research was conducted to determine if CDK6 binds to and promotes the degradation of other EYA family member proteins. Our data suggest that CDK6 binds to and degrades EYA3.

P1113**Board Number: B445****MECHANISTIC INSIGHTS TO CDK6-MEDIATED EYA2 DEGRADATION.**D. Kohrt¹, L. Placek¹, P. Hinds², H. Ford³, M. Gossel¹;¹Department of Biology, Connecticut College, New London, CT, ²Developmental, Molecular and Chemical Biology, Tufts University School of Medicine, Boston, MA, ³Pharmacology, University of Colorado School of Medicine, Aurora, CO

The D-cyclin activated kinase, CDK6, may only be essential for proliferation of specialized cell types, perhaps suggesting that it plays a role in differentiation. Our lab has shown that Cdk6 binds to and promotes degradation of the EYA2 protein. Here we investigate mechanisms of Cdk6-mediated EYA2 degradation. EYA2 is a member of the EYA family of transcriptional regulatory proteins that activate genes essential for the development of multiple organs. Eya proteins regulate cell proliferation and are misregulated in cancer. Our findings suggest that Cdk6 directly regulates the activity of the EYA2 protein. We propose that the Cdk6/Eya2 interaction affects fundamental properties of the cancer cell and may also function in normal development of some cell types.

P1114**Board Number: B446****Characterization of nuclear cyclin D1 interactions with replication regulatory elements in human cancer cells.**

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During normal cell cycle progression, cyclin D1 associates with CDK 4 and CDK 6 to regulate cells passage from G1 to S phase. In cancers cyclin D1 is either mutated, amplified or overexpressed altering cell cycle progression and may contribute to tumorigenesis. Cyclin D1 overproduction in the nucleus causes cancer cells to over-replicate portions of their genome and exhibit high levels of genomic instability. Here, we investigated mutant cyclin D1's role in the etiology of cancer. To that end, we characterized cyclin D1 expression patterns, cellular localization along with chromatin modifications, and replication profiles in cancer cells that exhibited cyclin D1 modifications. Our studies employed chromatin immunoprecipitation followed by whole-genome sequencing (ChIP-Seq), gene expression analyses, whole-genome replication profiling using asynchronous, and cell cycle fractionated cancer cells obtained by centrifugal elutriation. Since mutant cyclin D1 was shown to interact with members of the pre-replication complex in mice, we also asked whether replication patterns change in response to accumulation of nuclear cyclin D1. Cyclin D1 migrated to the nucleus in human hepatocellular carcinoma cells that were exposed to low doses of irradiation over a long time period (fractionated radiation). We also found that cyclin D1 was present in the nucleus of primary patient-derived leukemia cells. In those leukemia cells, the stabilized nuclear cyclin D1 associated with chromatin and exhibited sequence-specific interactions with activated promoters. These studies generated a whole-genome map of cyclin D1 chromatin binding patterns in cancer cells harboring mutated nuclear cyclin D1, allowing us to investigate if nuclear cyclin D1 binding sites co-localized with replication initiation sites and chromatin modifications. Future studies will probe possible alterations of replication patterns in cancer cells harboring mutated nuclear cyclin D1.

P1115**Board Number: B447****Investigating spindle checkpoint component expression levels in nasopharyngeal carcinoma tissue culture cells.**Y. Chu¹, S.C. Schuyler¹;¹Biomedical Sciences, Chang Gung University, Taoyuan, Taiwan

The spindle checkpoint is a mechanism contributing to the prevention of chromosome mis-segregation. De-regulation of the spindle checkpoint is thought to drive aneuploidy, potentially increasing the risk of tumorigenesis. A number of reports demonstrated alterations in the levels of spindle checkpoint components in cancer cells. Here, we focus on nasopharyngeal carcinoma (NPC), a cancer that remains a challenge to diagnose early. Our long-term goal is to characterize spindle checkpoint function in NPC cells. Our first objective was to investigate the expression levels of specific spindle checkpoint components, MAD1 and MAD2, and two other regulators of mitotic exit in NPC tumor cell lines TW-02 and TW-06 as compared to a normal nasopharynx epithelial cell line NP-69. We have measured the relative expression of MAD1, MAD2, p31comet and CUEDC2 mRNA by real-time quantitative PCR. We have observed that MAD2 expression levels appear to be aberrant. Our next goal is to perform similar analyses on mRNA isolated from NPC tumor biopsy samples.

P1116**Board Number: B448****Apical mistrafficking of epiregulin can be a driver event in epithelial cancers.**B. Singh¹, G. Bogatcheva¹, R.J. Coffey¹;¹Medicine (GI), Vanderbilt University Medical Center, Nashville, TN

The EGF receptor (EGFR) ligand epiregulin (EREG) is delivered preferentially to the basolateral cell surface of polarized MDCK cells. Recently, we showed that EREG basolateral trafficking is regulated by a conserved tyrosine residue within a YXXΦ motif (Y₁₅₆ERV) in its cytoplasmic domain. Interestingly, a Y156A substitution led to apical mistrafficking of EREG and transformation of polarized MDCK cells (PNAS 110: 8960-5, 2013). We have identified EREG mutations (R147stop) in human tumors that is predicted to disrupt the basolateral sorting motif of EREG; we now report that EREG mistrafficks to the apical surface in MDCK cells expressing the this mutation. To test the hypothesis that apical mistrafficking of EREG can be a driver event in epithelial cancers rather than a mere passenger, we have generated MDCK cells stably expressing, inducible (Tet-ON) wild-type and Y156A mutant EREG. Uninduced clones form normal appearing cysts in 3D Matrigel cultures, regardless of EREG status. Upon induction, however, only (Y156A)EREG-expressing cells form abnormal cysts with ectopic lumens and inward growth that correlates with transformation in Matrigel cultures, providing support for our hypothesis. Furthermore, we show that EGFR activity is required for EREG mistrafficking-induced ectopic lumen formation as preincubation with the irreversible EGFR kinase inhibitor, EKI-785 abrogates formation of lateral lumens. Using EREG cytoplasmic domain as a bait in a yeast-2-hybrid screen, we

have identified interacting proteins that could modulate polarized sorting of EREG. We have subsequently confirmed these interactions by co-immunoprecipitation. Under a separate inducible control (Cumate switch), we have now expressed these interacting proteins simultaneously with Tet-inducible EREG constructs, to test if their interaction alters EREG localization and subsequent ectopic lumen formation. Results from these experiments will be presented at the meeting. In summary, dynamic regulation of EREG localization (by interacting proteins) can mediate transformation induced by apical mistrafficking of EREG.

P1117

Board Number: B449

EGF promotes cell migration in 2D and 3D non-small cell lung cancer cultures mediated by Akt and Erk pathways.

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Lung cancer commonly exhibits ErbB1 gene mutation and amplification. ErbB1 encodes EGFR and its high expression in non-small cell lung cancer (NSCLC) has been associated with more aggressive disease and low response to therapy. Our group showed that HK2 cells have more ErbB1 gene copy number compared to A549 but lower EGFR expression. We also showed that EGF stimulus in NSCLC cells (A549 and HK2) grown in monolayer caused cell migration but not proliferation, independently of ErbB1 gene copy number or EGFR expression. The aims of this study are to investigate if Erk or Akt pathways are involved with regulation of EGFR-mediated motility and to establish a three dimensional culture (3D) to verify the effects of EGF, since 3D cultures is closer to *in vivo* conditions. 3D cultures were obtained by using cell suspensions in medium free of extracellular matrix elements submitted to rotation (20 rpm – alternating rotation to right/left) in non-adherent dishes at 37°C. A549 and HK2 cells formed spheroids. Western blotting analysis showed p-Akt and p-Erk expression, without EGF stimulation, in 2D and 3D cultures. The expression of these proteins increased after EGF stimulus. Exposure to AG1478, inhibitor of EGFR phosphorylation, decreased p-Akt levels only in 2D culture, but decreased p-Erk in monolayer and spheroids. We found p-EGFR expression without EGF stimulus in 3D. On the other hand, we did not observe EGFR phosphorylation in 2D culture without EGF stimulus, an important difference when we compare both systems of cell culture. Cell proliferation in spheroids was evaluated by cell cycle analysis and BrdU labeling by flow cytometry. Spheroids were stimulated with EGF or inhibited with AG1478 and no differences were detected in cell proliferation compared to control group. To investigate cell migration in monolayer we used wound healing assay, while sphere migration assay was performed in 3D cultures (spheroids were transferred individually to 24-well plates, treated with EGF or AG1478 and allowed to migrate; the diameter of migrating cells was measure). UO126 and LY294002 were added to inhibit, respectively, Erk and Akt pathways. Erk and Akt were involved in cell migration signaling in both cell lines cultured in monolayer. In 3D, cells exhibited higher rates of distant migration after EGF stimulation that was inhibited by AG1478. We could conclude that EGF did not induce cell proliferation but promoted cell migration in cells grown in 2D or 3D model. The migration of NSCLC cells induced by

EGF was mediated by Erk and Akt pathways. These results also provide new insights to culture NSCLC in a 3D model. Financial Support: FAPESP, CAPES and CNPq.

Cancer Therapy 2

P1118

Board Number: B450

Chromosomal copy number changes contribute to multidrug resistance in human cancer cells.

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Aneuploidy is an important and common feature of cancer, present in over 90% of solid human tumors. Many cancer cells become chromosomally unstable (CIN) - a state in which cells lose chromosome segregation fidelity - which can lead to persistent changes in karyotype and tumor heterogeneity. Importantly, CIN is associated with drug resistance and poor patient prognosis in most cancers. Acquired drug resistance is a serious contributing factor to the ineffectiveness of current therapies. However, it still remains unclear how CIN correlates with drug resistance. There are two competing models of how CIN relates to increased acquired drug resistance: (i) the selective karyotype model, which suggests that upon treatment, resistant karyotype(s) of the heterogeneous tumor will continue to proliferate and repopulate the tumor, while non-resistant karyotype(s) will die, and (ii) the intrinsic karyotype model, which suggests that CIN cells uniquely exhibit more dynamic cell signaling pathways, which make them more fit to survive chemotherapy treatments. Based on our findings, we hypothesize that CIN promotes multidrug resistance through the selective karyotype model. Our preliminary results support this hypothesis, as we see marked karyotype changes in multidrug resistant HeLa (R-HeLa) cells compared to parental HeLa cells. R-HeLa cells have increased mRNA and protein expression of the Multidrug resistant 1 (MDR1) ATP dependent efflux pump and exhibit high rates of lagging chromosomes. Through fluorescence in-situ hybridization and NanoString nCounter karyotyping, we discovered that R-HeLa cells exhibit stable karyotype changes with a gain of chromosomes 1 and 7, and a loss of chromosome 5. Interestingly, MDR1 is localized on chromosome 7. To investigate whether a single gene (MDR1) on a chromosome is sufficient for the selection of whole chromosome gain/loss, we have used targeted genome editing to insert MDR1 into the AAVS1 locus on chromosome 19 in HeLa cells. Confounding evidence for the selective karyotype model emanates from multiple karyotypes seen in resistant cell lines. However, the extent to which these chromosome changes lead to downstream protein changes or contribute to drug resistance is not understood. Therefore, we have begun to quantitatively analyze the proteomes and phosphorylation and ubiquitylation events of R-HeLa cell clones compared to non-resistant HeLa cells. This will allow us to better understand the downstream effects of karyotype changes in the context of CIN and multidrug resistance. These studies are crucial in understanding the cellular and molecular mechanisms of multidrug resistance.

P1119**Board Number: B451****Crosslink of cell cycle and cell migration.**H. Huang¹;¹Inst Molec/Cellular Biol-National Taiwan Univ, Taipei, Taiwan

Metastasis is the cause of 90% death from cancer, yet little is known about how it relates to other cellular processes. A recent dynamic investigation revealed that the metastasis promoter, Slug, is under temporal regulations during the course of cell cycle. This inspired the question of whether cell migration or invasion, and ultimately cancer metastasis, could also display a cell-cycle dependent pattern. With FUCCI (two-colored Fluorescent Ubiquitination-based Cell Cycle Indicator) and single-cell fluorescent microscopy, we were able to monitor cell cycle progression in individual cells while they migrate. We found that cells appeared to travel with highest speed in G1, as compared to the rest of the cell cycle. More specifically, cells tend to jump apart after cytokinesis. We hypothesize that the cortical pulling forces from the previous cell division could aid in cell migration. When we blocked spindle bipolarity with Kinesin-5 inhibitor (an antimetastatic drug), leaving cells with monopolar spindles, the high speed that peaked right after cell division was lost. Our results suggest that even if cancer cells resist killing from Kinesin-5 inhibitor, failed mitosis induced by the drug still have the benefit of preventing them to metastasize.

P1120**Board Number: B452****Probing and interrogating cell cycle control in MultiCellular Tumour Spheroids.**O. Mondésert¹, C. Frongia¹, C. Lorenzo¹, V. Lobjois¹, B. Ducommun¹;¹CNRS USR3505 ITAV, Toulouse, France

Major recent advances have recently been made in the understanding of the molecular pathways regulating the cell cycle machinery and their deregulations in cancer. These results open new avenues for the development of innovative antitumor pharmacological strategies targeting the cell cycle and its checkpoints.

MultiCellular Tumor Spheroid (MCTS) are now considered as invaluable models to study cancer cell biology and for the preclinical development of new antiproliferative drugs. To fully exploit the features of MCTS, it is essential to preserve the 3D regionalization level of analysis when investigating the effect of a drug. We will report here a breakthrough in the use of MCTS with new technological developments that can be used to explore the regionalization and the live dynamics of the effects of anticancer drugs in 3D. Spheroids expressing fluorescent cell cycle reporters (i.e. Fucci) and biosensors were engineered and used to monitor cell cycle arrest and DNA damage Response (DDR) pathway activation induced by chemotherapeutic agents. The kinetics and regionalized aspects of the response were investigated on genetically modified spheroids using innovative 3D imaging strategy (based on 3D light sheet microscopy). We will present our latest results on the characterization of the effects of several

anticancer drugs including DNA damaging agents alone or in association with checkpoint abrogating compounds.

This study opens new perspectives for original 3D assays dedicated to the identification of new original targets and compounds and for the investigation of the dynamics of the 3D response to novel antiproliferative agents.

P1121

Board Number: B453

Identification of evolutionarily conserved DNA Damage Response (DDR) genes that regulate response to cisplatin.

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Cancer treatment commonly involves the use of cytotoxic agents that damage DNA. Although these agents provide clinical benefit, tumors often develop resistance. The molecular mechanisms of resistance to one of the most common DNA-damaging agents, cisplatin, are not fully understood, although many known resistance mechanisms involve proteins functioning in the DNA Damage Response (DDR) pathways. A better understanding of the cellular response to DNA damage will probably give us possibility of identifying new therapeutic targets, providing new avenues for disease management.

To identify new regulators of DDR, we considered the fact that DDR involves multiple protein complexes and signaling pathways, some of which are evolutionarily ancient and conserved from yeast to humans. This is evidenced by the existence of yeast-human orthologs involved in certain DDR pathways. Based on this functional conservation, we hypothesized that thorough identification of genes regulating sensitivity to cisplatin and/or ionizing radiation in yeast may help to uncover human genes previously unknown to be involved in DDR. We first performed an extensive bioinformatics analysis, combining analysis of functional screens, gene expression response to DNA damage, protein interaction analysis, and other metrics. We then evaluated which genes shown to regulate resistance to DNA damage in *Saccharomyces cerevisiae* have human orthologs, identifying 122 candidates.

Squamous-cell carcinomas of the head and neck (SCCHN) are a heterogeneous group of cancers with a global incidence of 550,000 patients per year worldwide, and an annual mortality rate of 271,000. A major problem in treatment of SCCHN is an emergence of resistance to radio- and chemotherapy, which are frontline agents for the disease. We used siRNA knockdown of genes selected from the candidate gene set in a panel of SCCHN cell lines to identify those which regulate sensitivity to cisplatin. We have identified 20 genes that influence cisplatin resistance, and further explored their biological functions, analyzing their regulation of foci formation by phosphorylated histone protein γ H2AX (a sensitive marker for DSBs), cell cycle progression in presence and absence of cisplatin, and the activation and

expression of known DDR-associated signaling proteins. These results demonstrate the value of evolutionary modeling in generating new insight into therapeutic response.

P1122

Board Number: B454

In vitro and In vivo Efficacy of Diaminotiazoles is p53 Independent.

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Diaminotiazoles are a novel class of potential anticancer agents that show antimitotic and antiangiogenic properties. Earlier studies have shown that a lead diaminotiazole DAT1 (4-amino-5-benzoyl-2-(4-methoxy phenyl amino) thiazole) induced strong apoptotic response mostly due to triggering of Death Receptor 5 (DR5) mediated extrinsic pathway. We have studied the role of p53 and the upstream regulators of DAT1 action. We have also checked the toxicity and efficacy of DAT1 *in vivo* and confirmed the signaling pathway in xenograft tumours. p53 mutated and knocked out cell lines were used and signaling pathway was checked by western blotting. Acute and sub-acute toxicity studies were performed in Swiss Albino mice. Xenograft tumours were developed in SCID/NOD mice with subcutaneous injection of wild type colon cancer HCT116 cells and HCT116 cells with knocked out p53 gene.

Here, we found that DAT1 is cytotoxic to cell lines having dysfunctional p53 status through p53 independent upregulation of the death receptor DR5 followed by activation of caspase 8. It has also been found that DAT1 causes nuclear translocation and activation of ERK but not of p38 and JNK/SAPK. Acute toxicity study of DAT1 showed that there was no adverse effect of this compound up to 50 mg/kg. Further, in a sub-acute toxicity study, the serum ALP, ALT, AST, creatinine and BUN levels did not differ much from the control values which indicated that the drug is non-toxic to liver and kidney tissues. It was found that DAT1 was effective in causing reduction in both types of xenograft tumour growth in mice starting from a concentration of 10 mg/kg.

Tunnel assay and immunohistochemistry results clearly showed more apoptosis in tumour tissue that received DAT1 as compared to the tissue which received vehicle alone. The immunohistochemical results also revealed that DR5 and pERK expression were more in DAT1 treated tumour tissue as compared to the vehicle treated tissue while p38 and pJNK/SAPK levels were similar showing consistency with the *in vitro* results. Further, western blot studies of the tissue lysates also confirmed the immunohistochemistry results. Our findings thus show that the diaminotiazole DAT1 has minimal toxicity in mice and is highly effective in xenograft tumour models. Both *in vitro* and *in vivo* investigations show that ERK acts as an upstream regulator of DAT1 mediated apoptosis with p53 having little role. This study places diaminotiazoles as a highly promising anticancer agent even in tumours with inactivated p53.

P1123**Board Number: B455****Epigenetic repression of c-Myc P2 promoter by Sendai F-virosome mediated delivery of tumor specific shRNA in Hepatoma cells.**M.K. Zakaria^{1,2}, I. Khan¹, P. Mani³, D.P. Sarkar³, P. Chattopadhyay¹, S. Sinha²;¹Dept. of Biochemistry, All India Institute of Medical Sciences, New Delhi 110029, India, ²National Brain Research Centre, Manesar, Haryana 122050, India, ³Dept of Biochemistry, South Campus, University of Delhi, New Delhi 110021, India

Combinatorial association of a liver specific vehicular system with a therapeutic entity active only under neoplastic condition could make an ideal system for targeting hepatocellular carcinoma (HCC). Sendai virus home to hepatocytes based on the liver specific expression of asialoglycoprotein receptors (ASGPRs), which are recognized by viral fusion (F) protein's terminal galactose moiety. Sendai F-virosomes, derived from Sendai virus, are reconstituted viral envelopes with surface F-proteins. Removal of viral RNA genome and surface hemagglutinin neuraminidase (HN) proteins prevent their independent replication and non-specific/systemic interaction respectively. Transcriptional gene silencing (TGS) is a recent approach of RNAi involving dsRNAs. Unlike Post-transcriptional gene silencing (PTGS), TGS induces epigenetic silent state chromatin and DNA marks, leading to heritable and long term transcriptional repression of the target gene. Furthermore, it does not require continuous si/shRNA supply. ME1a1 binding site is required for maintaining active transcriptional state of the proto-oncogene c-Myc P2 promoter. Around 90% of the c-Myc transcripts are driven by its P2 promoter, making it a suitable candidate for targeted cancer therapy. Using such Sendai F-virosomal delivery, HCC activated alpha-fetoprotein (AFP) promoter was utilized, in combination with various tumor specific enhancers, to drive the expression of shRNA encompassing ME1a1 binding site, in order to induce TGS in hepatocarcinoma cells. The dual specificity of liver specific delivery with AFP promoter/enhancer driven expression of shRNA ensured that the TGS was activated only in transformed liver cells leading to apoptosis and decreased survival. The mechanistic study revealed that the extensive cancer cell death was as a result of specific histone modifications (H3K9 Di-methylated, H3K27 Tri-methylated and decreased H3 acetylation) and DNA methylation (CpG 8, 9 and 10) around the shRNA target locus, ultimately repressing the c-Myc P2 promoter. This was possibly due to the recruitment of histone deacetylases (HDACs) and DNA methyl transferases (DNMTs), by the shRNA, at the target site, since the shRNA failed to induce such epigenetic changes in the presence of DNMT/HDAC inhibitors. c-Myc down-regulation, by TGS, also led to the decrease of other pro-proliferative genes such as cyclin D3 and hTERT, suggesting a shift from neoplastic potential towards normal. This therapeutic system is potentially safe, since no interferon (IFN) response was generated by Sendai virosome/AFP promoter-enhancer – c-Myc shRNA expression system. Moreover, this could serve as an added advantage over other gene therapeutic approaches, since persistent c-Myc inactivation is required for HCC suppression. Additionally, this system could also be used to introduce genes specifically in embryonic liver and to tackle recalcitrant cancer cells with de-regulated c-Myc.

P1124**Board Number: B456****Characterization of an Inhibitor of the NF- κ B Signaling Protein NEMO.**S. Penix^{1,2}, T. Gilmore², A. Yeo²;¹Massbay Community College, Wellesley, MA, ²Molecular Biology, Boston University, Boston, MA

The Nuclear Factor kappa beta (NF- κ B) is a member of a family of eukaryotic transcription factors that are involved in inflammatory response, developmental processes, cellular growth, and apoptosis. It is persistently active in a number of disease states such as cancer, chronic inflammation, asthma, and neurodegenerative diseases and has been heavily implicated in their onset. Activation of NF- κ B requires the cytoplasmic phosphorylation and later degradation of interferon kappa-beta alpha (IKBA), these events release of NF- κ B for entry into the nucleus where it would finally serve its role as a transcription factor. Because of its possible role in chronic and acute inflammation as well as the onset of cancer, NF- κ B modulation has often been considered as a likely candidate for therapeutic agents. Therefore, many groups have sought to identify compounds that can inhibit NF- κ B and/or its pathway for diverse therapeutic and regulatory purposes. In this project, we characterized a recently synthesized compound (identified as 9644) that has been shown to inhibit NF- κ B signaling by disrupting the NF- κ B essential modulator (NEMO) and interferon kappa kinase (IKK) protein-to-protein interaction. The NEMO/IKK protein complex is required for activation of the I κ B kinase complex in canonical NF- κ B signaling and is directly responsible for the phosphorylation and degradation of I κ BA. It is important to note that the activation of the NEMO/IKK complex itself is product of the activation of a tumor necrosis factor (TNF) receptor, a protein also entangled in the networks of cancer cell biology and inflammation. Previous analysis of the compound 9644 through crystallography have hinted that the mechanism of action of compound 9644 does entails a direct interaction with a cysteine residues located within and around the CC1 and CC2 domains of NEMO. For our analysis we have induced point mutations within clones of NEMO cDNA to induce point mutations and subsequent substitutions of the cysteine residues for alanine residues within the CC1 and CC2 domains to confirm the specific residue that 9644 binds. After confirmation of the mutations through use of restriction enzymes and PCR, the different NEMO mutants were added to plasmid backbones to for transfection into human embryonic kidney cells (A293T) culture, expression and analysis of the effects that the 9644 compound would have in them, the formation of the NEMO/IKK complex and activation of NF- κ B. Analysis of protein extracts with Western Blotting showed that replacing all the cysteine residues in the indicated region (7xALA NEMO construct) does reduce the action of 9644 while still permits the formation of the NEMO/IKK complex. We hope to expand our analysis in the future to pinpoint the specific residue responsible for the action of the compound 9644.

P1125**Board Number: B457****Lung cancer cells after irradiation indicate viability and malignancy dependent on activating transcription factor 5.**S. Ishihara^{1,2}, M. Yasuda³, A. Ishizu⁴, H. Haga^{1,2};

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Radiotherapy is a major therapeutic way for treating malignant tumors. However, a part of cancer cells after irradiation survives and repopulates tumors that indicate malignant phenotypes correlated with poor prognosis. It is not exactly known how cancer cells survive and regenerate malignant tumors after irradiation. We here show that activating transcription factor 5 (ATF5) enhances viability and malignant phenotype, such as tumorigenesis, cell growth, and invasiveness, in lung cancer cells after irradiation. We established a transgenic A549 human lung adenocarcinoma cell line overexpressing ATF5 (A549-ATF5). A549-ATF5 cells indicated high viability comparing with control A549 cells after 10 Gy irradiation. Next, we examined the relationship between the expression of ATF5 and the cell cycle. ATF5 was highly expressed during the late G1 phase to S phase in A549 cells. Furthermore, at the late G1 phase, the cells indicated higher viability after irradiation than the M phase. In addition to the viability after irradiation, A549-ATF5 cells also displayed high ability of cell growth and invasiveness comparing with control A549 cells in vitro. A549-ATF5 cells also showed higher ability of tumorigenesis and invasiveness than control cells in vivo. Moreover, A549 cells that survived after irradiation indicated greater expression of ATF5 than control A549 cells and showed high invasiveness dependent on ATF5 expression. We also revealed that ATF5 enhances the invasiveness in A549 cells by promoting the expression of integrin beta1, which contributes to cell-matrix adhesion, and dephosphorylation of myosin regulatory light chain, which regulates cellular contractile force. These results suggest that lung cancer cells which highly express ATF5 dependent on cell cycle survive after irradiation, and as a result, regenerate more malignant tumors than non-irradiated lung cancer cells.

P1126**Board Number: B458****Elucidating the Role of Centrosome Amplification in Tumorigenesis.**M.S. Levine¹, A.J. Holland¹;

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Centrosomes are the major microtubule organizing centers of animal cells and play an important role during mitosis where they organize the poles of the bipolar microtubule spindle apparatus upon which chromosomes are segregated. Extra copies of centrosomes frequently result in errors in spindle assembly that give rise to chromosome missegregation and the production of aneuploid daughter cells

with an abnormal chromosome content. Almost one hundred years ago, Theodor Boveri proposed that centrosome amplification could contribute to tumorigenesis. Since then, supernumerary centrosomes have been identified as a consistent feature of aneuploid tumors. Moreover, centrosome amplification is found early in the development of some hematological cancers and solid tumors and has been shown to correlate with tumor grade, proliferative index and the level of genomic instability. However, despite the large body of circumstantial evidence linking extra centrosomes to the development of cancer, it remains unclear whether supernumerary centrosomes actively contribute to tumorigenesis or arise as a byproduct of cellular transformation.

The serine/threonine kinase Polo-like kinase 4 (Plk4) is a master regulator of centrosome copy number, and its overexpression leads to the production of supernumerary centrosomes. To test the in vivo consequence of centrosome amplification in the absence of other defects, we have developed a novel mouse model in which Plk4 can be reversibly overexpressed using a doxycycline-inducible promoter. Using this animal model, we show robust centrosome amplification can be induced in multiple primary cell types in vitro and in vivo. We have observed that overexpression of Plk4 leads to supernumerary centrosomes in 25%, 50%, and 80% of bone marrow cells, mammary epithelial cells, and mouse ear fibroblasts, respectively. Moreover, we observed that centrosome amplification is lethal early in life, but well tolerated in young adult animals. We will describe our ongoing efforts using this model to analyze the consequence of centrosome amplification in tumor development.

P1127

Board Number: B459

The Effect of Nuclear Import Inhibition on Ovarian Cancer Cells.

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BACKGROUND Karyopherins are a family of protein receptors responsible for transporting cargo molecules across the nuclear membrane. Previous studies have shown that KPNA2, a member of this protein family promotes cell proliferation and tumorigenicity, and that its over-expression significantly correlates with poor prognosis in ovarian cancer (OvCa). KPNA2 acts in concert with KPNB1 and together they import proteins carrying the nuclear localization signal. KPNB1 has also been shown to be expressed in other cancers such as cervical and esophageal, and its inhibition using both siRNA and a small molecule inhibitor of nuclear import, C43 result in cancer cell death. C43 is a small molecule identified in silico as a potential KPNB1 inhibitor and showed inhibitory effects on KPNB1 associated nuclear import. The aim of this study is to investigate KPNB1 expression and nuclear import inhibition using C43 in OvCa. **METHODS** Western blot analysis and qRT-PCR were used to measure KPNB1 protein and mRNA expression in ten OvCa cell lines. The effect of inhibiting nuclear import using a small molecule C43 on OvCa cell biology was investigated, including cell survival; and KPNB1 cellular distribution using immunofluorescence and nuclear-cytoplasmic separation. KPNB1 expression was examined in response to platinum-induced cellular stress, and a combination treatment of cisplatin with

the nuclear import inhibitor was explored. **RESULTS** KPNB1 was found to be highly expressed in all the OvCa cell lines examined at both the mRNA and protein levels. Treatment of OvCa cells with the nuclear import inhibitor C43 induced OvCa cell death. Immunofluorescence and nuclear/cytoplasmic fractionation showed C43 was able to decrease nuclear localization of KPNB1 in OVCAR4 and OVCAR8 cell lines. Furthermore, sub-lethal doses of cisplatin treatment resulted in increased KPNB1 expression within one hour of treatment, suggesting that KPNB1 may be involved in platinum-induced early stress-response. Pre-treating OvCa cells with C43 to inhibit nuclear import via KPNB1 increased the sensitivity to cisplatin treatment. **CONCLUSION** Our data shows that the nuclear receptor KPNB1 is highly expressed in OvCa cells and that inhibiting nuclear import results in OvCa cell death. In addition, KPNB1 appear to have a role in the platinum induced early stress response in OvCa. As drug resistance and toxicity is a major problem in treating OvCa, our data suggest that a combination treatment of nuclear import inhibition via KPNB1 and cisplatin may increase the sensitivity of OvCa cells to cisplatin.

P1128

Board Number: B460

Mediator Kinase Module is a transducer of Oncogenic Wnt/Beta-catenin signaling.

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CDK8 is a colorectal cancer (CRC) oncoprotein whose amplification-dependent overexpression identifies a significant subset of colorectal cancer patients with poor prognosis. Mechanistically, Cyclin C (CycC)-dependent CDK8 kinase activity drives tumorigenesis by regulating beta-catenin transcriptional activity. Accordingly, inhibition of CDK8 kinase function offers a promising therapeutic approach for CDK8-overexpressing colorectal cancers. Cyc-CDK8, along MED12 and MED13, comprise a discrete 4-subunit "kinase" module within Mediator, a conserved multiprotein interface between gene-specific transcription factors and RNA Polymerase II. Here, we identify a network of physical and functional interactions within the Mediator kinase module critical for oncogenic Wnt/beta-catenin signaling. We show that beta-catenin binds directly to MED12 in Mediator, which in turn activates CycC-dependent CDK8 kinase activity and beta-catenin target gene induction. MED12-dependent CDK8 activation occurs through a direct interaction involving the MED12 N-terminus and a phylogenetically conserved surface groove on CycC. Mutagenic disruption of the MED12/CycC interface inhibits CDK8 kinase activity and beta-catenin target gene expression, and concomitantly impairs CRC cell proliferation and clonogenicity. These findings identify the MED12/CycC interface as a critical transducer of oncogenic Wnt/beta-catenin signaling and a potential therapeutic target in colorectal and other CDK8-driven malignancies.

P1129**Board Number: B461****A snapshot of pan-cellular PTM in response to Sorafenib mediated multi-kinase inhibition.**M. Bell¹, C. Loch¹;¹Research and Development, AVMBioMed, Malvern, PA

More fundamentally than regulating specific functions or cascades, PTM affords proteins the mechanical complexity necessary to make human cells function. We believe that specifically modified forms of proteins are what conduct the cells business, and that by monitoring these “proteoforms” we can gain unique insight into cellular physiology. To test this, we treated HepG2 hepatocellular carcinoma cells with the multi-kinase inhibitor Sorafenib, and observed pharmacodynamic changes to PTM across the cellular proteome. Our novel approach, referred to as Snapshot Proteomics, revealed changes in phosphorylation to known targets of this drug. Intriguingly, we also observed changes to proteins whose roles make them strong candidates for mediating the clinical side effects of Sorafenib treatment. We also observed that the changes mediating the therapeutic response were far from the most dramatic ones observed, suggesting other, unexpected proteins as better putative pharmacodynamic biomarkers. Finally, we found that manipulating cellular phosphorylation pharmacologically had dramatic and interesting effects on other PTM pathways as well, ubiquitin in particular. This confirmed the already established crosstalk between these pathways, and supports our contention that no PTM occurs in a vacuum.

P1130**Board Number: B462****K20E, an Oxidative-coupling Compound of Methyl Caffeate, Exhibits Anti-angiogenesis Activities through Down-Regulations of VEGF and VEGF Receptor-2.**C. Wu¹, C. Pan^{1,2}, . Lin³;¹Pharmacy, Taipei Medical University, Taipei, Taiwan, ²Pharmacy, Taipei Medical University, Taipei, Taiwan, ³Pharmacy, China Medical University, Taichung, Taiwan

Anti-angiogenesis is one of the most popular clinical interventions for applying on cancer chemotherapy. A new synthesized derivative of methyl caffeate, named as K20E, was used to investigate its anti-angiogenesis activity and possible pharmacological mechanisms. Anti-angiogenesis effects of K20E were evaluated in the experiments of murine allograft tumor model and Matrigel plug assay as well as cell models in the human umbilical vascular endothelial cells (HUVECs) and the LLC1 lung cancer cells. Our results suggested that K20E suppressed tumor growth in the allograft tumor model and exhibited anti-angiogenesis activity in Matrigel plug assay. Besides, HUVEC viability can be significantly reduced through inducing cell cycle arrest at G2/M phase and apoptosis. Cell migration, invasion, and tube

formation of the HUVECs were also markedly suppressed by K20E treatment. K20E largely down-regulated the protein expression of vascular endothelial growth factor (VEGF) in the LLC1 cancer cells. Besides, the expression levels of VEGF receptor-2 (VEGFR-2) and its downstream signaling cascades (PI3K-AKT-mTOR and MEK1/2-ERK1/2) as well as the expression and activity of gelatinases were also evidently reduced in the HUVECs treated with K20E. Inversely, K20E can up-regulate the expression levels of p53 and p21 proteins in the HUVECs. Based on these results, our study suggested that K20E possessed antitumor effect by anti-angiogenesis which is associated with reducing VEGF expression in the cancer cells as well as decreasing the expression and activation levels of VEGFR-2 and its downstream signaling cascades in the vascular endothelial cells (VECs).

P1131

Board Number: B463

Carcinoma-specific inhibition of cell proliferation by down-regulating farnesoid x receptor expression.

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Farnesoid X receptor (FXR) is a pivotal factor maintaining bile acid homeostasis and highly expressed in the liver, kidney, intestine, and adrenal gland. We have previously revealed that FXR controls the proliferation of hepatocellular carcinoma cells: FXR stimulates cell proliferation by suppressing the p16/INK4a expression whereas downregulation of the FXR stimulates the expression of CDK inhibitors p16/INK4a and p21/Cip1, resulting in the suppressed cell proliferation (Fujino T, et al. J.Biochem. 2012). In the present study, we investigate whether or not FXR exerts carcinoma-specific cytotoxicity toward hepatocellular carcinoma cells and renal adenocarcinoma cells.

Hepatic cells: The treatment of HepG2, human hepatocarcinoma cell line, with FXR siRNA transcriptionally elevated the level of p16/INK4a and p21/Cip1 expression resulting in the inhibition of cell proliferation. By contrast, in human hepatocyte-derived cell line Fa2N-4, FXR knockdown post-transcriptionally reduced p16/INK4a and p21/Cip1 expression resulting in the stimulation of cell proliferation. Proteasome inhibitor treatment restored FXR knockdown-caused down-regulation of p16/INK4a and p21/Cip1 in Fa2N-4 cells, indicating the down-regulation of p16/INK4a and p21/Cip1 is proteasome-dependent.

Renal cells: The treatment of ACHN, human renal adenocarcinoma cell line, with FXR siRNA transcriptionally elevated the level of p21/Cip1 expression resulting in the inhibition of cell proliferation. By contrast, in human renal cell line HK-2, FXR knockdown transcriptionally reduced p21/Cip1 expression resulting in the stimulation of cell proliferation.

In conclusion, FXR negatively regulates the cell proliferation of hepatocellular carcinoma cells and renal adenocarcinoma cells, while it stimulates that of normal hepatic and renal cells. Our study may contribute the development of anti-cancer medicine with minimal side-effects.

P1132**Board Number: B464****Using Targeted Molecular Imaging Agents (TMIA) to Evaluate Differences between Two- and Three-Dimensional Cell Culture Cancer Models.**H.D. Ophardt¹, A.M. Butera¹, H.F. Schmitthenner², I.M. Evans¹;¹GSOLS, Rochester Institute of Technology, Rochester, NY, ²SOCAMS/ Center for Imaging Science, Rochester Institute of Technology, Rochester, NY

The goal of this research is to recreate and understand the three dimensional (3D) organoid which constitutes cancer, the King of the Maladies in order to develop better targeted molecular imaging agents (TMIA). To create a 3D cancer model, cancer cells are embedded in a matrix whose signaling contributes to malignant progression of the cancer cells. To further understanding of cancer cells and metastases, the human lung adenocarcinoma cell line A549 and the murine brain endothelial cell line, bEnd3, were cultured using traditional two-dimensional methods as well as several three-dimensional matrices including, Matrigel, Geltrex, and Collagen type I. An ever increasing body of literature indicates significant differences in cell morphology, gene expression, proliferation, migration and many other cellular properties between 2D and 3D cultured cells, with 3D culture more accurately representing that which is observed within live animal models in vivo. Fluorescent microscopy on a Leica TCS SP5 confocal microscope was used in conjunction with multiple fluorescent dyes including NucBlue, Tubulin Tracker Oregon Green, and Mitotracker Red, targeting the nucleus, microtubules, and mitochondria respectively. In addition, the targeted molecular imaging agent (TMIA) Cy5.5-RGDyk conjugate, synthesized at RIT, which specifically targets $\alpha\beta3$ integrins and has been shown to be over expressed on the surface of some cancer cell strains, was also employed. Our results show differences between the properties of 2D and 3D cell culture systems which may be important in the way cancer cells metastasize and spread throughout the body of a cancer patient. We also observed that the TMIA agents utilized penetrated 3D cancer models and stained cells buried inside the spheroid tumor model. We conclude that the use of 3D cellular models which mimic more closely in vivo tumors should facilitate development of Targeted Molecular Imaging Agents (TMIA) and result in molecules which target to metastatic tumors illuminating their presence, size, and structure thus allowing better treatment and enhanced cancer survival.

P1133**Board Number: B465****Novel and facile fluorescence microscopy method for detection of intracellular galectin-3 inhibition by small molecules in vitro.**G. Sharma¹, J. Stegmayr^{1,2}, M. Carlsson^{1,3}, U.J. Nilsson⁴, S. Oredsson², H. Leffler¹;¹Department of Laboratory Medicine, Lund University, Lund, Sweden, ²Department of Biology, Lund University, Lund, Sweden, ³Department of Cellular and Molecular Medicine, University of Copenhagen, Copenhagen, Denmark, ⁴Department of Chemistry, Lund University, Lund, Sweden

Galectins are intra- and extracellular proteins that bind to β -galactosides and are implicated in a broad range of cellular functions such as cell proliferation, cell adhesion, and apoptosis with consequences in cancer and inflammation. As possible treatments of such diseases, we have developed high affinity inhibitors of galectin-3, the most studied galectin. However, there is no method till date to determine whether a therapeutic agent (galectin inhibitor) will reach and bind to intracellular galectin. Taking this into account, there was an unmet need to develop highly specific method capable of detecting intracellular inhibitor effects on galectin-3. Hence, we analysed the effects of a galectin-3 inhibitor (bis-{3,3'-deoxy-3,3'-[4-(3-fluorophenyl)-1*H*-1,2,3-triazol-1-yl]}-1,1'-sulfanediyl-di- β -D-galactopyranoside) on an intracellular effect directly shown to depend on galectin-3 carbohydrate interaction – the accumulation of galectin-3 around disrupted intracellular vesicles. Treatment of cells with Glycylphenylalanine-2-naphthylamide (GPN), or Leucine-Leucine-Omethyl (L-L-OMe), or amitriptyline was used to induce disruption of lysosomes and accumulation of galectin-3 around them, observed by immunofluorescence microscopy as puncta. Pretreatment of the cells with the inhibitor inhibited this accumulation of galectin-3, recorded as decreased formation of puncta in a dose and time dependent manner.

P1134**Board Number: B466****Fluorescence-based co-culture of normal and cancerous cells as an indicator of cancer therapeutic effects.**H. Matsui¹, M. Tamura¹, J.K. Tanaka¹, Y. Miwa¹;¹Faculty of Med, Univ Tsukuba, Tsukuba, Japan

Comprehensive evaluation of cancer therapeutic effects is difficult in vitro because of the need to distinguish the main effects from the side effects with in data. This difficulty cannot be overcome by comprehensive evaluation involving monoculture. For a solution of this problem, we established two cell lines which are the GFP-expressing rat gastric mucosal cell (RGM-GFP) and the Kusabira orange-expressing cancerous RGM cell (RGK-KO), and use of these, we tried fluorescence-based co-culture with both of RGM-GFP and RGK-KO. An origin of RGK-KO was established from RGM by an exposure of chemical carcinogen, therefore, the native gene background was same among both of cells. RGM-GFP

and RGK-KO should be an ideal pair of cells for comprehensive evaluation in some cancer fields. We evaluated major cancer therapy such as chemotherapy and X-ray treatment using this co-culture. These results clearly distinguished the cancer-selective toxicity. While Adriamycin induced a cell death against both of cells, 5-FU induced it against RGK-KO mainly. X-ray treatment also showed a cancer-selective toxicity under our experimental condition. We also tried a three-dimensional co-culture with both of cells. RGM-GFP was spread and created the monolayer on a matrigel, while, RGK-KO invaded into matrigel. Furthermore RGK-KO on the monolayer of RGM-GFP also invaded into matrigel through the layer. As a conclusion, we developed the fluorescent co-culture of normal cells and cancer cells for evaluation of cancer therapeutic effect, especially cancer-selective toxicity. We could distinguish main effects from side effects in anti-cancer drug and X-ray treatment using the co-culture system. The three-dimensional co-culture with matrigel showed potential as a promising method for use in biological and pharmaceutical science fields.

P1135

Board Number: B467

Double edge sword: Atmospheric gas plasma as a source of protection against tumor development for normal cells and as a killer for tumor cells.

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Background: Atmospheric gas plasmas (AGP), which is source of reactive oxygen and nitrogen species is able to activate anti-oxidant system in normal cells. Furthermore AGP can selectively induce apoptosis in cancer cells, offers a promising alternative approach to conventional therapies which have unwanted side effects such as drug resistance and toxicity. However, the mechanism of AGP-induced anti-oxidant system activation in normal cells and cancer cell death is unknown. **Purpose of the study:** The aim of this study was to explore the intracellular mechanism, how AGP induced anti-oxidant system in normal non-transformed cells and apoptosis in cancer cells without effecting normal cells. **Experimental procedures:** Mice (BALB/c-Fox1nuAusb) were used in this study. AGP treated solution was injected I/V into mice. Melanoma cancer cell lines were injected S/C into AGP treated or untreated mice to develop tumor. Tumor tissues and normal melanocytes were used to study the effect of AGP by measuring the apoptosis, intracellular ROS/GSH/NO production, intracellular gene expression by RNA Microarray and qPCR, immunoblotting and ELISA and enzymatic activity of kinases.

Results: In this study, mice injected with AGP-treated solution were resistant to develop tumor compared to untreated mice which develop melanoma tumor in 2 weeks. Gene expression array study showed that antioxidant genes like Srx, NQO1 and AKR1C1 were up-regulated in AGP treated mice normal melanocytes cells compared to untreated mice. Knockout of anti-oxidant activator Nrf2 in mice blocked the AGP ability to activate antioxidant enzymes like Srx in mice and revert mice ability for tumor development. On the other hand, mice without AGP treatment developed melanoma. AGP treatment of tumors reduced the volume of tumors. Gene expression array studies show that genes from TNF (tumor-necrosis factor) pathway were mostly differentially up-regulated. Further studies showed AGP activated

ASK1 kinases downstream of TNF pathway were involved to induce apoptosis in tumor cells. This data show, activation of anti-oxidant system in mice can prevent tumor development and also AGP can kill tumor cells by inducing apoptosis without effecting normal cells. Future study will focus to attain long-term activation of anti-oxidant system in the animals to resist tumor development. Further experiments will be conducted to explore the exact mechanism involved in AGP-induced antioxidant system defence mechanism against tumor development.

Conclusion: The evidence for Nrf2 dependent anti-oxidant system activation and resistance against tumor development after AGP-treatment can be used as a new tool for cancer preventive therapy.

P1136

Board Number: B468

Development of (+)-negamycin-derived ester-type prodrugs promoting premature termination codon-readthrough and its application for suppressing cancer cell growth.

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Duchenne muscular dystrophy (DMD) is the most severe form of dystrophinopathy caused by a mutation in the dystrophin gene, which codes an important structural protein dystrophin within muscle tissue. About 10-20% of this congenital disease is caused by nonsense mutations possessing the premature termination codons (PTCs). One promising approach against this mutation is a readthrough drug strategy, which is comprised of drugs that allow the translational machinery to skip PTCs, resulting in the production of full-length functional dystrophin. (+)-Negamycin **1**, which was a dipeptidic antibiotic containing a hydrazide structure, was reported that (+)-**1** restored dystrophin expression in the muscles of mdx mice, an animal model of DMD (Arakawa, M. *et al.*, *J. Biochem.*, 2003). Hence, the potent derivatives of (+)-**1** would be a promising therapeutic candidate for diseases caused by nonsense mutations. In our structure-activity relationship study through the reporter assay, we discovered more potent derivatives 3-*epi*-deoxynegamycin (Taguchi, A, *et al.*, *ChemMedChem*, in press) and TCP-112 without significant anti-microbial activities. These derivatives were particularly effective in the readthrough of a TGA-type nonsense mutation. Moreover, we synthesized derivatives of TCP112 focused on its C-terminal part (N-methyl *hydrazinoglycine* part) and found that C-terminal ester derivatives (TCP-169) exhibited a more potent readthrough activity. TCP-169 significantly suppressed the proliferation of cancer cells caused by nonsense mutation. These results suggested that (+)-negamycin derivatives were promising platform for the development of readthrough drug toward nonsense mutation-mediated genetic diseases.

P1137**Board Number: B469****Effect of Let-7a on Human Malignant Melanoma Cells.**G. Yang¹, J. Jiang¹, L. Wang¹, Y. Ma¹, Y. Yang¹;¹Emporia State University, Emporia, KS

Objective: The goal of this study is to examine the effect of Let-7a on the proliferation and invasiveness of human malignant melanoma cells and identify the gene(s) that Let-7a targets to affect the growth and metastasis of melanoma cells. **Methods:** The effect of Let-7a on proliferation, migration and invasion was measured by cell viability assay and Wounding healing assays, and Transwell migration assay, respectively. The expressions of MITF, and MAP4K3 genes at protein level were measured by Western blot. Cell cycle progression was determined by flow cytometer. **Results:** Let-7a displayed a significant ($p < 0.05$) inhibitory effect on the proliferation, migration and invasion on SKMEL-28 and WM1552C human melanoma cell lines. In addition, Let-7a induced cell cycle arrest at G1 phase. MITF gene was down-regulated by Let-7a in melanoma cells. **Conclusion:** Let-7a inhibited the growth, migration and invasion, also induced cell cycle arrest at G1 phase in human malignant melanoma cells. The expression of MITF was suppressed by Let-7a in SKMEL-28 and WM1552C melanoma cell lines. Taken together, these data suggests Let-7a act as tumor suppressors in melanoma cells and have the potential to function as a novel therapeutic small molecule against human malignant melanoma.

P1138**Board Number: B470****Investigating the effects of tellurium compounds on melanoma cell lines.**A.A. Martens¹, S.C. dos Santos¹, R.L. Cunha², G.M. Machado-Santelli¹;¹Departamento de Biologia Celular e do Desenvolvimento, Universidade de São Paulo, Sao Paulo, Brazil,²Centro de Ciências Naturais e Humanas, Universidade Federal do ABC, Santo André, Brazil

Melanoma genetic background is very heterogeneous, with mutations that compromise different signalling pathways in each case, which poses great difficulty to obtain a wide spectrum drug that would be reliable for these different mutations. Tellurium compounds have shown to act as anticancer drugs in a still unclear manner – they possess a marked redox potential, which appears to react with thiols inactivating proteins important to the survival of cancer cells. In this work, we evaluated the action of two enantiomeric organotelluranes on a melanoma panel, comprised of 4 human melanoma cell lines (HT-144, SK-Mel-19, SK-Mel-28, SK-Mel-147), murine melanoma cell line B16F10, and NGM (human nevus cell line) as a control. Previous experiments with these compounds showed inhibition of melanoma lung metastases in vivo, which seems to be associated with decreased migration and invasion capability in a concentration-dependent manner. Hence, it is important to study the cellular effects of these compounds. We decided to investigate their effects on cytoskeleton and cell cycle alterations, as well the induction of nuclear abnormalities. Cytoskeleton alterations varied, going from mild microfilaments hyperstabilisation to heavy disarray, with cell roundness and cortical distribution of

microfilaments and microtubules. Cell cycle disturbances seemed to be related to increased frequency of G2/M cells. Deeper analyses still have to be done to understand which signalling pathways intermediates are influenced by the action of these organotelluranes.

P1139

Board Number: B471

Prevention of the development of GST-dependent cisplatin resistance in a model of human H526 small cell lung cancer (SCLC) by ethacrynic acid and selenite.

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Tumor cell resistance to the chemotherapy drug cisplatin (CP) has been associated with many mechanisms, including increased activity of glutathione-s-transferase (GST). While many studies have focused on reversing CP resistance, we are exploring its prevention, using models to mimic its development and to screen preventative agents. We previously designed an in vivo model of CP resistant small cell lung cancer (SCLC) using human H526 cells growing as xenografts in mice. We have now initiated cell cultures from two of these tumors: from an untreated control tumor (designated C-H526), and from a tumor (designated R-H526) pretreated with the dose of CP (1.5 mg/kg) that resulted in resistance to subsequent treatments. Tumors were homogenized, cells suspended and cultures maintained in RPMI with 15% FBS. Replicate dishes were exposed to either PBS or CP concentrations, counted before and after exposure and cell counts expressed as mean % of pre-dose counts. Following 36 hr exposure to 10 uM CP, 86.3% of R-H526 cells survived, compared to 45.7% survival of C-H526 cells. Thus cells retained the relative resistance of the tumors from which they were derived. We then measured GST activity as a function of GS-DNB conjugate production: it was nearly twice as high (1.04 activity units) in the resistant R-H526 cells as in the C-H526 cells (0.53 activity units). This suggests that inhibitors of GST activity, such as ethacrynic acid (EA) or selenite (which prevents GST-associated drug resistance in ovarian cancer cells) might prevent CP resistance in SCLC. To screen these potential preventative agents, we designed an in vitro model in which CP resistance was induced by pretreating H-526 cells with low, subtoxic concentrations of CP. After 36 hr, these cells were exposed for 24 hr to a high, toxic (15 uM) dose of CP. In response to this high dose CP, control cells pretreated with PBS decreased by 58.7%, whereas cells pretreated with low dose (7 uM) CP actually increased by 25.12%, indicating the development of resistance. To determine their prevention activity, subtoxic doses of EA or selenite were added to cultures 6-24 hrs prior to CP-pretreatment. Replicate cultures were pretreated with test agent alone or with PBS. Co-pretreatment with 40 uM EA caused a cell count decrease of 48.13% in response to high CP dose. The efficacy of EA confirms that this resistance is dependent on increased GST. Co-pretreatment with 1.5 uM selenite also prevented CP resistance, resulting in a 49.06% decrease in cells in response to high dose CP. This study suggests that increased GST activity is the mechanism of cisplatin resistance in our SCLC models, and that co-pretreatment with either EA or selenite prevents the development of this resistance.

P1140**Board Number: B472****Promising activity of Diaminotiazoles in overcoming multidrug resistance in cancer cells and xenograft tumor models.**S. Vasudevan¹, S.A. Thomas¹, K.N. Rajasekharan², S. Sengupta¹;¹Cancer Research Program-3, Rajiv Gandhi Centre for Biotechnology, Trivandrum, Kerala, India,²Department of Chemistry, University of Kerala, Trivandrum, Kerala, India

Antimitotic drugs are powerful chemotherapeutic agents, but their potentials are limited by neurotoxicity and development of resistance. Overexpression of drug efflux pumps along with target specific modulations and improper apoptotic machinery are found in tumors. Many small antimitotic agents are effective in multidrug resistant tumors, mainly because they elude the efflux pumps. However, eventually they also evoke resistance. It is important to find out agents that are potent in resistant tumors and to understand the resistance mechanisms against small molecules so that judicious combinations can be devised. We have investigated the efficacy of diaminotiazoles, a novel class of small, antimitotic, potential anticancer compounds, in multidrug resistant cancer cells. Also, our work compares the resistance mechanisms aroused in cells against small and large molecules.

Model resistant cell lines were raised against the clinically used antimitotic drug Taxol and a lead diaminotiazole, 4-amino-5-benzoyl-2-(4-methoxy phenyl amino) thiazole (DAT1). Xenograft tumors were derived from them in SCID mice. *In vivo* antitumor activity of DAT1 was tested in these multidrug resistant tumor models. Apoptosis was measured by chromatin condensation, caspase 3 and PARP cleavage. Cell cycle analysis was done by flow cytometry. Immunoblotting was conducted to analyze the expression of various resistance associated proteins: P-glycoprotein, beta tubulin isotypes, apoptotic and checkpoint proteins. Primary cells were derived from these xenograft tumor models and cytotoxicity studies were conducted.

Diaminotiazoles induced mitotic arrest leading to perturbation in checkpoint proteins like AuroraA, AuroraB, Mad2 and Bub1; eventually triggering resistant cells to undergo apoptosis. They could overcome the overexpression of P-glycoprotein, beta II and III tubulin isotypes and imbalance in the pro and apoptotic protein ratio. These proteins were also studied in xenograft tumors developed in SCID mice. P-glycoprotein overexpression was found to be the prominent resistance mechanism in SCID mice bearing Taxol resistant cells. Cytotoxicity studies with the primary cells derived from SCID mice bearing DAT1 resistant cells showed that their resistance against DAT1 diminished, and was lost quickly with passaging; indicating the transient as well as specific nature of DAT1 resistance.

Thus, diaminotiazoles showed promising antitumor activity in multidrug resistant cancers both *in vitro* and *in vivo*. We have also found that resistance against antimitotic drugs with higher size is broad-spectrum and multifactorial. On the contrary, resistance against small molecules is transient and target specific.

P1141**Board Number: B473****Proteomic profiling identified multiple short-lived members of the central proteome as the direct targets of the addicted oncogenes in cancer cells.**T. Qi¹, W. Zhang², Y. Luan¹, F. Kong¹, D. Xu¹, G. Cheng¹, Y. Wang³;¹Central Research Laboratory, The Second Hospital of Shandong University, Ji Nan, China, ²Department of Medical Imaging, The Second Hospital of Shandong University, Ji Nan, China, ³Central Research Laboratory, Jinan Central Hospital Affiliated to Shandong University, Ji Nan, China

Oncogene addiction is an unexplained phenomenon in the area of cancer targeted therapy. In this study, we have tested a hypothesis that rapid apoptotic response of cancer cells following acute inhibition of the addicted oncogenes is because of loss of multiple short-lived proteins whose activity normally maintain cell survival by blocking caspase activation directly or indirectly. It was shown that rapid apoptotic response or acute apoptosis could be induced in both A431 and MiaPaCa-2 cells, and quick down-regulation of 17 proteins, which were all members of the central proteome of human cells, was found to be associated with the onset of acute apoptosis. Knockdown of PSMD11 could partially promote the occurrence of acute apoptosis in both MiaPaCa-2 and PANC-1 pancreatic cancer cells. These findings indicate that maintaining the stability of central proteome may be a primary mechanism for addicted oncogenes to maintain the survival of cancer cells through various signaling pathways, and quick loss of some of the short-lived members of the central proteome may be the direct reason for the rapid apoptotic response or acute apoptosis following acute inhibition of the addicted oncogenes in cancer cells. These findings we have presented can help us better understand the phenomenon of oncogene-addiction and may have important implications for the targeted therapy of cancer

P1142**Board Number: B474****Ultrasonication as a Means to Degrade Chitosan.**C. Carnegie¹, C.D. Johnson¹, F. Akindoju¹, E. Williams¹, J.M. Stone¹, A. Oki², E.C. Regisford³, L.E. Carson¹;¹Cooperative Agricultural Research Center, Prairie View AM University, Prairie View, TX, ²Chemistry Department, Prairie View AM University, Praire View, TX, ³Department of Biology, Prairie View AM University, Prairie View, TX

Many drug delivery studies have been performed using polymeric material like poly(ethylene glycol) and poly(ethyleneimine). However, there is a need to study polymers that are less toxic to the body. In recent years, chitosan, a naturally occurring, biodegradable, linear cationic polysaccharide that is composed of β -(1-4) linked glucosamine and N-acetyl-D-glucosamine have been studied. Over time different methodologies are being used to successfully break apart the long chains of chitosan, one such method is ultra sonication. This study identifies the effect of ultrasound technology on natural sonication condition, chitosan dissolved in acetic acid. The ultrasonic frequencies used lead to

the process being known as ultra sonication or ultra-sonication. Fourier Transform Infrared Spectroscopy (FTIR) indicated no change in the characteristics of Chitosan, showing that between the 10 minutes, 20 minutes and 30 minutes timed ultra sonication experiments Chitosan showed no significant change in its chemical and physical properties. Thermo gravimetric Analysis (TGA) showed that 20% weight loss temperature of chitosan derivatives' was maintained between all the samples listed above and reiterated th.at there was no change in the physical and chemical properties of Chitosan.

P1143

Board Number: B475

Cytotoxicity on human breast cancer cells caused by a vanadium salt.

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Vanadium and its compounds have been investigated for anti-cancer effects for a long time. The effects of vanadium on tumour growth inhibition have been investigated in T-cell leukaemia and B-cell lymphoma, as well as in cancers of the testis, the uterus, the kidney, the lung, the throat and the oesophagus [1]. Vanadium compounds are stated to have pharmaceutical effects on different types of cancer cells [2]. Vanadyl sulphate is found to cause single and double-strand breaks in the DNA in HeLa cells and prostate adenocarcinoma [3], also growth inhibition of rat hepatoma cell line [4]. At the present study we aimed to investigate the cytotoxic effects of vanadyl sulphate on human breast cancer cells. A stock solution of the agent (in distilled water) was prepared and for further dilutions fresh culture medium was used. In order to detect cytotoxicity for 24 hours, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H tetrazolium bromide) colorimetric assay was applied with the 50, 60, 70, 80, 90, 100, 110, 120 μ M concentrations of vanadyl sulphate. The viability percentages of the treated cells 68.2, 62.0, 59.01, 50.36, 47.89, 47.82, 38.2 and 29.0, respectively. IC50 concentration for 24 hours was determined as 85 μ M. According to our results vanadyl sulphate is found to be highly cytotoxic on human breast cancer cells. This result may show the anticarcinogenic potential of the agent and may be encouraging for further investigations in drug designing systems for cancer treatment.

P1144

Board Number: B476

An antibody that inhibits Wnt signaling in colorectal cancer cell lines.

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The Wnt pathway is a highly conserved pathway that controls many developmental processes. In the absence of Wnt ligand, cytoplasmic b-catenin is degraded by a destruction complex assembled by the protein Axin and the tumor suppressor adenomatous polyposis coli (APC). Within this complex b-catenin is phosphorylated by glycogen synthase kinase 3 (GSK3) and targeted for ubiquitin-mediated

degradation. Activation of the Wnt pathway occurs upon binding of Wnt ligand to the seven-pass transmembrane protein Frizzled (Fz) and the low-density lipoprotein receptor-related protein 6 (LRP6). The formation of a Wnt-Fz-LRP6 complex promotes LRP6 phosphorylation, which binds to the b-catenin degradation complex. Binding of phosphorylated LRP6 to the b-catenin degradation complex leads to inhibition of both b-catenin phosphorylation and degradation. b-catenin subsequently accumulates in the cytoplasm, enters the nucleus, and activates a Wnt-transcription program. Mutations in the APC or b-catenin genes leading to constitutive activation of the Wnt pathway occur in over 80% of human colorectal cancers. Our lab has developed a monoclonal antibody (mabLRP6) that targets the coreceptor LRP6. Preliminary studies have shown the effectiveness of this antibody in inhibiting Wnt signaling *in vivo*, decreasing the expression of Wnt target genes in mice. Also, mabLRP6 blocks ligand-dependent Wnt activation in mammalian cells. Furthermore, mabLRP6 inhibits Wnt signaling in the colorectal cancer cell lines SW480 and DLD1, which are mutant for APC and exhibit constitutive Wnt signaling. This evidence suggests that mabLRP6 acts as a specific Wnt inhibitor and is a potential treatment for Wnt-driven tumors.

P1145

Board Number: B477

Podoplanin (PDPN) presents a unique target to inhibit tumor cell growth and motility.

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Cancer is a leading cause of death. In fact, cancer killed over 8 million people around the world in 2012. Over 90% of these cancer deaths are due to metastasis, which results from tumor cell migration and invasion. Specific cancer biomarkers need to be identified in order to effectively target these motile cells. The transmembrane glycoprotein receptor podoplanin (PDPN) promotes tumor cell motility and metastasis in many aggressive cancers. Indeed, PDPN has emerged as a prime cancer biomarker and chemotherapeutic target. Here, we describe how PDPN can be targeted to inhibit the growth and motility of melanoma and oral cancer cells. PDPN has a short intracellular domain of 9 amino acids which include two conserved serine residues. PDPN also has a large extracellular domain that is extensively O-glycosylated with α 2,3-sialic acid linked to galactose. We are developing novel methods to target the intracellular and extracellular domains of PDPN to combat cancer progression. For example, we have found that some activators of protein kinase A can induce phosphorylation of the intracellular serine residues of PDPN to inhibit tumor cell migration. In addition, we have found that Maackia amurensis seed lectin (MASL) can target the extracellular domain of PDPN to inhibit tumor cell growth, migration, and tumor progression in cell culture and animal models. Furthermore, we utilized live cell

imaging to find that PDPN expression can be modulated in cancer associated fibroblasts to inhibit neighboring tumor cell migration and survival. Thus, reagents can be used to target PDPN from inside of the cell and outside of the cell to inhibit tumor cell migration and combat cancer progression. This work illuminates novel strategies designed to exploit PDPN as a functionally relevant biomarker and chemotherapeutic target.

P1146

Board Number: B478

ABCC3 expression is up-regulated in prostate gland after androgen deprivation.

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Nowadays, prostate cancer is one of most important disease in males. Different treatments have been used to stop cell proliferation and to reduce cell survival of prostate cancer cells using as target the androgen receptor (AR) inhibition. However, after androgen blockade, many prostate cancers keep growing and developing under in the androgen-deprived environment. Our laboratory has tried to figure out new molecules and phenomena present in prostate gland after androgen deprivation in an attempt to find new therapeutic targets. In this work, comparing ventral prostate gland of Wistar rat (90 days old) from control group (CT) with those from surgically castrated rats (Cas) for 3 days, we figured out by DNA microarray (n=3) that the gene *Abcc3* was up regulated in Cas (eight-fold increase). The ABCC3 protein is a pump, member of family that promotes multidrug resistance. In cancer, this pump is responsible for drug efflux, rendering the cells resistant to chemotherapy. Therefore, the ABCC3 protein is an interesting target, because it might contribute for the resistance of prostate cancer to the treatments. The validation of microarray data was done by qRT-PCR, which showed a 35-fold increase in Cas as compared to the CT group (n=3). Using immunofluorescence, ABCC3 was found in the baso-lateral plasma membrane. To check if the ABCC3 is modulated by androgens, the functional experiment was done using RWPE-1, a normal prostate cell line, which expresses ABCC3 protein in the plasma membrane. The RWPE-1 cells were grown on matrigel layer without cypionate of testosterone (CT); with 10 nM of androgen for 96h (T); 48h with androgen and 48h without it (T48). When the cells were 90% confluent, it was added 100 uM in their media of two different substrates: one group as a negative control with rhodamine (cationic - not pumped by ABCC3) and another group with calcein (anionic - pumped by ABCC3). Loading was allowed for 1 hour. Afterwards, the media was changed and left for 20 minutes at 37°C and then collected. The media was changed and collected every 20 minutes up to 100 minutes. The collected media was read in a fluorimeter to quantify how much of substrate was pumped by RWPE-1 cells under testosterone conditions. After the quantification, we observed no rhodamine was pumped while calcein was better pumped by cells from T48 group than T and CT. Using *in silico* analysis of the ABCC3 proximal promoter region, revealed two putative AR binding site, suggesting there is a mechanism of *Abcc3* regulation in the prostate gland under androgen modulation. This study points to

the fact that androgens might regulate the expression of protein pumps to promote drug-resistance in the absence of androgens and also points to a molecular relationship between *Abcc3* and AR.

P1147

Board Number: B479

Biological Testing of Novel Telomerase Inhibitors.

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Normal cells are limited in the number of times they can divide by the caps on the ends of their chromosomes, called telomeres. These caps are supposed to become degraded over time, eventually signaling the cell to die when they become too short. During the summer of 2013, three novel compounds were made via synthesis of cinnamoyl chloride derivatives. These three compounds all contain active sites that are identical to those identified on BIBR 1532, a known telomerase inhibitor, with one key difference in the element attached to the aromatic ring. These three compounds were tested for anticancer properties on metastatic prostate cancer cell lines. Their efficacy will be compared against that of BIBR 1532 to determine if this novel compound would prove to be an adequate cancer treatment. If these compounds prove to be telomerase inhibitors, it would be a breakthrough as to how BIBR 1532 functions, and could potentially lead to a more effective cancer treatment. While the compounds were tested using metastatic prostate cancer cells, these potential treatments have applications in both breast and pancreatic cancers as well.

P1148

Board Number: B480

ANALYSIS OF N-GLYCOME OF INDUCED HEPATOCELLULAR CARCINOMA: A PRE-CLINICAL STUDY.

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Hepatocellular carcinoma (HCC) is one of the most common types of cancer worldwide. Adequate markers are not present for the diagnosis of HCC. Glycans are emerging sensitive and simple potential biomarkers for various diseases including cancer. The aim of the present study is to evaluate qualitative and quantitative changes in N-linked glycosylation of proteins that occur in response to HCC in animal model of liver cancer. Liver tissue samples of 2 groups of rats- 1) normal (non-tumor-bearing) rats; 2) tumor-bearing rats; were collected and the liver lysates were used for biochemical and GlycanMap[®] analyses. Briefly, GlycanMap[®] analysis is high-throughput assay that provides a structural and

quantitative readout of protein-associated glycans using a unique, automated 96-well assay technology coupled to matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and custom bioinformatics. Histopathology studies were also done to ensure the development of HCC in rat models. The glycomics analysis revealed 5 novel glycans that showed statistically significant differences between the normal and tumor rats. There was increase in high mannose structures in HCC rats compared to normal. Importantly, HCC rats showed increase in tumor-associated carbohydrates. The branched glycans were also increased in association with HCC. Those fluctuations in glycans correlated well with mutations of enzymes involved in glycan biosynthetic pathway, hence making altered glycans potential and reliable biomarker for HCC. Further study is needed to validate these findings in HCC cancer patients.

P1149

Board Number: B481

Molecular basis underlying resistance against Mps1/TTK inhibitors.

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Mps1/TTK is an essential dual specificity kinase, with an essential role in mitotic checkpoint signaling and proper alignment of the chromosomes. Mps1/TTK kinase has emerged as a potential target in cancer therapy and several Mps1 small molecule inhibitors have been described to date that exhibit promising anti-proliferative activity in human cancer cells. Among these compounds, NMS-P715, MPI-0479605, Mps-BAY1, Mps-BAY2a, Mps-BAY2b, and Mps1-IN-3 showed promising results in pre-clinical studies with rodent xenograft models. Phase I clinical trials for one of the Mps-BAY compounds in breast cancer treatment have been initiated recently. The emerging application of Mps1/TTK inhibitors in cancer therapy raises the question whether cancer cells can develop resistance to these drugs. Here, we identified and characterized a new potent and selective ATP-competitive Mps1/TTK kinase inhibitor. In addition, we identified four point mutations in the catalytic domain of Mps1/TTK that gave rise to inhibitor resistance but retained wild-type catalytic activity. Interestingly the single mutations confer resistance to only a subset of inhibitors, which seems non-specific for inhibitor scaffolds. Our studies predict that drug-resistant Mps1/TTK cells are likely to arise through the acquisition of mutations in the ATP pocket of the kinase that prevent the stable binding of the inhibitors. In addition, our results provide insight for future drug design to evict possible resistance mutations during clinical treatment.

P1150**Board Number: B482****In-Vitro Rescue and Recovery Studies of Human Melanoma (BLM) Cell Growth, Adhesion and Migration Functions after Treatment with Mifepristone (RU-486).**D.C. Leder¹, P. Ramaraj²;¹Biochemistry, A.T.Still University, Kirksville, MO, ²Biochemistry, A.T.Still University, Kirksville, MO

Our previous work with progesterone and its receptor antagonist RU-486 showed a significant inhibition on human melanoma (BLM) cell growth *in-vitro* (1,2,3). The mechanism of inhibition of cell growth by RU-486 was due to apoptosis (4). Inhibition of apoptosis by pan-caspase inhibitor (CI) showed a partial rescue in cell growth. This observation prompted us to check the effect of CI on other in-vitro functions such as adhesion and migration, as there were lot of cells floating in the medium. Partial rescue in cell growth also prompted us to investigate recovery of those in-vitro functions on their own after removing Ru-486 and CI. Hence, the aim of the study was to compare in-vitro functions of **RU-486 treated, CI rescued** and **recovered** human melanoma cells. Human melanoma (BLM) cells were treated with RU-486 or RU-486 + CI (20 μ M). After 24 hrs of treatment, cell growth was quantitated by MTT assay, adhesion was quantitated by crystal violet binding assay and migration by drill assay.

Twenty-four hrs of RU-486 (50 μ M) treatment resulted in 50% cell growth compared to untreated control cell growth (100%). But, addition of 20 μ M of CI to the cells, partially rescued cell growth to 67%. After 48 hours of recovery, treated cells recovered cell growth to 70%, whereas rescued cell recovered cell growth to 95%. When both cells were allowed to recover for a second 48 hrs, treated cells showed a cell growth of 82% and rescued cell 97%. CI rescued cells showed complete recovery of cell growth, whereas treated cells showed significant recovery. RU-486 (50 μ M) also resulted in 60% adhesion compared to control cell adhesion (100%). But CI rescued adhesion to 75%. When treated and rescued cells were allowed to recover for first 48 hrs, treated cells adhesion rose to 85%, whereas CI rescued cells adhesion rose to 97%. When the two group of cells were allowed to recover for a second 48 hrs, treated cells adhesion was 90% and CI rescued cells adhesion was 98%. Both treated and rescued cells recovered close to control level of adhesion. Migration drill assay showed that RU-486 (50 μ M) decreased migration to 26% compared to untreated control (100%). But CI rescued migration partially to 38%.

In conclusion, Ru-486 treatment decreased in-vitro cell growth, adhesion and migration functions of human melanoma (BLM) cells. So, Ru-486 has the potential to be an anticancer agent for melanoma. When apoptosis was blocked by CI, there were partial rescue in cell growth, adhesion and migration functions. But when the cells were allowed to recover, rescued cells recovered completely back to control level, whereas treated cells recovered significantly close to control level as reported in the recovery study of other metastatic cancer cell lines (5).

P1151**Board Number: B483****Inhibition of OV2008 Ovarian Cancer Cell Proliferation in the presence of Oleoyl Ethanolamide and Chlorpyrifos-Oxon.**J. Ricker¹, C. Kramer², P. Bagavandoss²;¹Integrative Health Studies, Kent State University @ Stark, North Canton, OH, ²Biological Sciences, Kent State University @ Stark, North Canton, OH

We have previously shown that both the acylethanolamide oleoylethanolamide (OEA) and its metabolically stable analog AM3102 (N-[(1R)-2-hydroxy-1-methylethyl-9Z-octadecenamide) induce apoptosis in OV2008 ovarian adenocarcinoma cells independent of PPAR- α receptor signaling pathway. This cytotoxicity was reversed in the presence of α -tocopherol, indicative of reactive oxygen species (ROS) involvement in cell death. We have also shown that palmitoyl trifluoromethyl ketone (PTK), independent of its inhibitory effect on phospholipase A2, enhanced the toxicity of OEA (ASCB 2012, Abstract #888). The enzyme neutral cholesterol ester hydrolase 1 (NCEH1) has also been shown to be a target of trifluoromethylketones (Nat Biotechnol 21:687, 2003). Furthermore, inhibition of NCEH1 leads to reduced migration of SKOV3 ovarian cancer cells in vitro and their tumor growth in vivo (Chem Biol 13: 1041, 2006). Therefore, in this study, using chlorpyrifos-oxon (CPO), a potent inhibitor of the enzyme NCEH1 (Toxicol Sci 91:166, 2006), we explored the relationship between OEA and NCEH1 in the OV2008 ovarian cancer cell line. Cytotoxicity was observed in response to both OEA (IC₅₀: 14-21 μ M) and CPO (IC₅₀: 48-50 μ M). When the cells were incubated with both compounds together, cytotoxicity was enhanced (OEA IC₅₀: 8-9 μ M; CPO IC₅₀: 15-19 μ M) with evidence of weak synergism. However, Western blot analysis of OEA and CPO treated cells indicated, when compared with control, no decrease in the expression of NCEH1. Live cell labeling with dihydroethidium showed an increase in superoxide by about 10% in the presence of OEA. CPO itself did not cause an increase in ROS. Live cell fluorescence microscopy using MitoSOX Red also revealed an increase in superoxide anion in mitochondria in the presence of OEA. The results of our study suggest that one mechanism by which OEA induces cytotoxicity in OV2008 cells is via superoxide generation. However, how additional toxicity is induced in the presence of both OEA and CPO needs further exploration (This undergraduate student research was supported by funds from the University Research Council).

Regulatory and Noncoding RNAs

P1152

Board Number: B485

Dependence of stress granule formation on microtubules supports the idea of stress granule specific “glue” arising in stress conditions.

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Stress granules are RNP-containing aggregates arising in cells under stress conditions and accumulating small ribosomal subunits, mRNPs and translation initiation factors. Previously, microtubules were shown to be involved in stress granule formation, although their role remained obscure. In this study, we found that stress granules' formation did depend on microtubules at low concentration of a stressing agent (arsenite) but did not depend on them at high arsenite concentration. However, at any arsenite concentration stress granules formed slower in cells with disrupted microtubules and were smaller. We found also that stress granules' formation was not directly coupled with protein synthesis inhibition, e.g. at low arsenite concentration protein synthesis was inhibited though stress granules did not form. We built a numerical model based on the suggestion that stress granules and their progenitors (presumably, 48S pre-initiation complexes) diffuse in cytoplasm and merge on contact. They could also move along microtubules by either linear diffusion or active transport. In such a model, if we proposed that each stress granules' and/or progenitors' contact resulted in their merging, the stress granules' formation did not depend on microtubules. Decreased probability of merging upon contact made the system dependent on microtubules, and microtubules served as assembly scaffolds since they had 100% probability to bind stress granules and/or their progenitors. In our model the contribution of active transport along microtubules to stress granule formation was variable and also dependent on overall merging probability rate. This model also suggests that there is an apparent optimum of microtubules for assembly of stress granules, which can explain the phenomenon of stress granules forming a ring in cytoplasm. The data support the concept of “glue” arising in stressed cells that binds stress granule components together.

P1153**Board Number: B486****Slicing activity of the *C. elegans* Argonaute CSR-1 tunes the expression of germline genes to control embryonic divisions.**A. Gerson-Gurwitz¹, S. Wang¹, K. Oegema¹, A. Desai¹;¹Ludwig Institute for Cancer Research, La Jolla, CA

The *C. elegans* Argonaute CSR-1 interacts with 22G small RNAs that are antisense to germline-expressed transcripts. Inhibition of CSR-1 leads to defects in spindle assembly and chromosome segregation in early embryos, resulting in penetrant embryonic lethality. Despite its robust mRNA slicing activity *in vitro*, a role for CSR-1 in restricting target expression has been disfavored. Here, we show that the CSR-1 depletion phenotype in early embryos closely resembles phenotypes associated with reduced microtubule assembly. Replacing CSR-1 with an engineered mutant lacking slicing activity further revealed that all early embryonic phenotypes associated with CSR-1 inhibition are directly related to its slicing function. Inspection of prior gene expression data indicated that the message encoding the microtubule depolymerase MCAK/KLP-7 is elevated 1.6 fold when CSR-1 is inhibited, suggesting that increased MCAK/KLP-7 activity could be responsible for the microtubule assembly defect. Immunoblotting indicated that MCAK/KLP-7 protein levels were elevated ~3.5-fold compared to controls, and reducing MCAK/KLP-7 levels restored microtubule and spindle assembly in CSR-1-inhibited embryos. Thus, MCAK/KLP-7 levels need to be precisely controlled by CSR-1 because even modest overexpression of this potent microtubule depolymerase leads to a dramatic phenotype. This result suggested that CSR-1, which binds 22G-small RNAs derived from essentially all germline transcripts, also tunes the expression levels of other germline proteins. To test this, we performed quantitative immunoblotting to measure the levels for 44 germline proteins for which antibodies are available. The majority of the tested targets were elevated between 1.5-3.5 fold in the presence of the CSR-1 slicing activity mutant, and the extent of elevation correlated with the abundance of CSR-1-bound 22G RNAs. We conclude that the CSR-1/22G-small RNA system functions to tune the expression of a large number of germline-expressed genes and that this control is essential for embryonic divisions. We speculate that evolution and/or organismal physiology employs this system to precisely control protein levels by adjusting 22G RNA levels for particular transcripts.

P1154**Board Number: B487****Expression levels of miR-454-3p and miR-33b in low birth weight and high birth weight neonates*.**S.P. Rodil-García¹, L.A. Salazar-Olivo¹;¹División de Biología Molecular, Instituto Potosino de Investigación Científica y Tecnológica, San Luis Potosí, Mexico

Recent advances in understanding the etiology of metabolic disorders have focused attention on the mechanisms related to its developmental origins, in which birth weight has long been used as an indicator [1–3]. MicroRNAs (miRNAs) are non-coding RNA molecules that act as post-transcriptional regulators. They have been proposed as early biomarkers since they are involved in the regulation pathways of several diseases [4,5]. To determine whether differences exist in miRNAs expression levels from neonates with different birth weight, we recovered miRNAs from neonatal screening Guthrie cards obtained from normal, low and high birth weight Mexican neonates and quantified their expression using an LNA-probe based stem-loop RT-qPCR. MiR-454-3p was over-expressed in low birth weight infants (p

P1155**Board Number: B488****The expression and the intracellular localization of $\Delta E2$ which is novel splice variant of Disrupted-In-Schizophrenia 1 (DISC1).**Y. Miyatake¹, M. Taniguchi², S. Matsuzaki^{1,2}, M. Tohyama^{1,3,4}, T. Manabe⁵, T. Katayama¹;¹Division of Molecular Brain Science, United Graduated School of Child Development, Osaka University, Suita City, Osaka Pref., Japan, ²Graduated School of Medicine, Osaka University, Suita City, Osaka Pref., Japan, ³Osaka Prefectural Hospital Organization, Osaka City, Osaka Pref., Japan, ⁴Research Institute of Traditional Asian Medicine, Kindai University, Osakasayama City, Osaka Pref., Japan, ⁵Institute for Comprehensive Medical Science, Fujita Health University, Toyoake City, Aichi Pref., Japan

Alternative splicing (AS) events contribute to generate distinct mRNAs from same primary transcript. Recently, it has been reported that some causes of diseases are abnormal AS events. On the other hand, disrupted in schizophrenia 1 (DISC1), is neural development related factor, acts on several pathways of brain development such as migration, proliferation, and neurite outgrowth of neurons. Moreover, it has been indicated that the DISC1 AS variants and its mRNA expression of patients with schizophrenia (SZ) differ from those of controls.

In this study, we identified the novel Disc1 variant that lacks exon 2 ($\Delta E2$) in mice brains by using RT-PCR assay. Northern blot analysis using Locked Nucleic Acid (LNA) probe showed the expression of $\Delta E2$ mRNA, clearly different from full length Disc1 (FL). LNA probe is an artificial nucleic acid probe which is bridged RNA and has features which are the capable of single nucleotide discrimination, the increased

target specificity, and the increased the thermal stability of duplexes. It thought suitable LNA probe was utilized for the detection of AS variants. As the result of northern blot assay, the expression of $\Delta E2$ in cortex and hippocampus increased in the developmental stage dependent manner, whereas the expression of FL at the developmental stage from embryonic day 15 to postnatal 8 weeks. Moreover, immunocytochemical analyses of ectopic- $\Delta E2$ -Flag and -FL-Flag in SK-N-SH cells were performed to reveal the subcellular localization of epitopic- $\Delta E2$ and -FL-Flag in cultured cell lines. The ectopic- $\Delta E2$ -Flag protein distributed in nucleus and in cytoplasm with fiber shape, and these cytoplasmic immunoreactivities were co-localized with F-actin. On the other hand, the ectopic-FL-Flag protein localized predominantly in cytoplasmic granule in cultured SK-N-SH cells.

These data show differences of the expression and localization patterns between the $\Delta E2$ and FL. Hereafter, conducting further performance analysis of $\Delta E2$ may solve the new role of Disc1, and it may be useful for the elucidation of the mechanisms of SZ development.

P1156

Board Number: B489

Investigation of dysregulated pathways involving long non-coding RNA (lncRNA) and gene expression of NR4A1 in myelodysplastic syndromes and acute leukemia.

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The advent of whole transcriptome sequencing technologies revealed that most of the human genome is transcribed, producing a large repertoire of non-coding RNAs (ncRNAs). Among the different types of non-coding RNAs, long non-coding RNAs (lncRNAs) are arbitrarily defined as transcripts composed of more than 200 nucleotides. Recently, the number of studies describing the functionality and diversity long non-coding transcripts mapped in intronic and intergenic regions has increased dramatically. Whole transcriptome analysis shows that up to 70% of coding genes have antisense counterparts, and several examples of local regulation have been described in the literature. They are known to participate in epigenetic regulation at different levels and alter mRNA stability of coding genes. In this context it is not surprising that long non-coding RNAs are emerging as key players in carcinogenesis. We hypothesized that lncRNAs encoded in locus previously associated with hematopoietic malignancy may regulate hematopoiesis and development of malignancy. In this study, we identified an antisense non-coding transcript in the NR4A1 gene locus. The tumor suppressor NR4A1 has been associated with the regulation of apoptosis and proliferation pathways in various tumors and shows a reduced expression in AML. Additionally, the abrogation of this locus in murine models caused the development of myelodysplastic syndrome. The expression of NR4A1 and the antisense non-coding was evaluated by qRT-PCR and we observed a reduction of NR4A1 gene expression in AML and MDS patients with P

P1157**Board Number: B490****A novel multiplex assay for miRNA detection and profiling using barcoded hydrogel particles.**M.A. Santos¹, M. Bader¹, M.H. Hsu¹;¹Cell Biology, EMD Millipore, Temecula, CA

miRNAs play key roles in regulating gene expression. Abnormal miRNA expression has been observed in many human diseases, making miRNAs a new class of diagnostic biomarkers. Traditionally, miRNAs are studied using applications such as real-time PCR, microarrays, and by northern blot analysis. All of these applications do have their benefits; however, each platform also carries a few disadvantages.

Here we present a unique miRNA multiplex detection assay using barcoded hydrogel particles that can be detected on any existing flow cytometer. By using this porous, bio-inert, fluorescently encoded hydrogel particles, we can achieve up to 70 plex detection using the combination of two different concentrations of the barcode dye Cy3 at each ends of the bar-shaped particle; a 2nd dye is used for detection of miRNA target that hybridizes to the capture probe, hybridization of the specific target miRNA to capture probe will enable the templated ligation of the target to a universal biotinylated adapter, which will fluoresce upon binding to streptavidin PE-Cy5.

In this study, we validated the performance of this novel multiplex miRNA profiling assay using a variety of miRNA samples. We tested for miRNA target specificity and sensitivity by profiling the let-7 family of miRNA targets, which differs by only one nucleotide. Additionally, we have established the dynamic range and sensitivity of the assay to achieve 3-4 log dynamic ranges as well as sub-attomole sensitivity for detection. As a cross validation study, we performed RT-PCR to confirm our results, indicating that this novel microRNA detection system is a valid model for miRNA profiling and screening across a variety of testing samples.

P1158**Board Number: B491****miRNA-148/152 family members concordantly target genes important for tumor progression and chemoresistance.**D.J. Oliver¹, H. Ji¹, S. Lee², M. Karthikeyan³, H. Valafar⁴, M. Shtutman¹;¹Drug Discovery and Biomedical Sciences, University of South Carolina, Columbia, SC, ²Biology, University of South Carolina, Columbia, SC, ³Chemistry and Biochemistry, University of South Carolina, Columbia, SC, ⁴Computer Science and Engineering, University of South Carolina, Columbia, SC

To date, over 1800 human miRNAs have been identified (miRBase 21) targeting virtually every mRNA. The means by which miRNAs exert their effect on protein translation is usually through (i) inhibition of

translation, (ii) mRNA de-adenylation, or (iii) direct mRNA degradation. Which mechanism of translational repression occurs depends on miRNA/mRNA specific interactions. In addition, it is well known that each miRNA can target multiple mRNAs and conversely, many mRNAs are regulated by multiple miRNAs. The complexity and sensitivity of regulatory networks that these interactions can produce is astounding and require genomic scale experiments to tease out. Here we present our work using RNA sequencing (RNA-seq) to identify networks of genes regulated by the miRNA-148/152 family. RNA-seq provides several advantages for studying miRNA regulation networks including whole genome scale and identification of de-adenylation targets, direct degradation targets, and genes downstream of direct miRNA targets. The miRNA-148/152 family is a highly conserved miRNA family made up of three miRNAs - miR-148a, miR-148b, and miR-152. miRs-148b and 152 are encoded in the COPZ1 and COPZ2 genes, respectively while miR-148a is transcribed independently. The sequence of these miRNAs differ in only 2 base pairs (both outside of the classic seed region) making target prediction difficult. In addition, the miRNA-148/152 family members are differentially regulated during cancer progression. Specifically, miRs-148a and 148b are often maintained or upregulated while miR-152 becomes silenced in many cancers. Interestingly, analysis of RNA-seq data from 2 different prostate cancer cell lines over-expressing each of the three miRNA-148/152 members (8 cell lines total) shows that the majority of regulated genes are concordantly regulated rather than differentially regulated as expected from cancer progression data. These results suggest that the miR-148/152 family may behave in a rheostat model to regulate target mRNAs. Our RNA-seq data suggests that the miR-148/152 family of miRNAs are regulating genes and pathways involved in cancer development and progression, including EMT markers, EGFR family receptor signaling and DNA repair. Direct targets of miRNAs, identified through HITS-CLIP experiment and an analysis of miRNA dependent pathways, based on combined HITS-CLIP and RNA-seq results will be presented.

P1159

Board Number: B492

Differential expression of miR9, miR21 and miR34a in women with breast cancer.

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The discoveries of sensitive and specific minimally invasive biomarkers that can be used to detect early breast cancer are necessary. MicroRNAs (miRs) are involved in many cellular functions, and changes on their expression have been associated with cancer development and progression. Our aim was to determine the expression of miR9, miR21 and miR34 in sera of healthy women and patients with breast cancer at different stages. MicroRNAs were isolated from 200 µl of serum by using a miRNeasy serum/plasma kit (Quiagen). The MicroRNAs expression was analyzed by real-time PCR using the miRCURY LNA Universal RT microRNA PCR kit (Exiqon). Synthesis of cDNA was performed using specific primers for miR9, miR21 and miR34a. MiR16 was used as endogenous control. Expression was analyzed

using the ΔC_t method. Our findings demonstrate a differential expression of miR9 with respect to different stages and women without breast cancer; whereas miR21 and miR34a do not show different expression. These data suggest that miR9 might be used as marker to prognostic on late stages. This work is supported by a grant from ICYTDF(224/2012).

P1160

Board Number: B493

Sub-cellular localization of novel long non-coding RNAs in mouse early B cell differentiation.

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Long non-coding RNA (lncRNA) transcripts are longer than 200 nucleotides (nt) and have no apparent protein-coding open reading frame longer than 100 amino acids. Long suspected to be products of pervasive, accidental, or unregulated transcription, lncRNAs have recently been implicated in regulation of differentiation (mESC, cardiogenesis, adipogenesis), cellular pathways (immune response, X chromosome inactivation, senescence), and nuclear architecture (paraspeckles, splicing speckles, X chromosome architecture). With the exception of a few noted examples, very little is known about the functions of lncRNAs, particularly in primary models or developmental systems. Therefore, we sought to identify novel lncRNAs in early B cell development in mouse and functionally characterize their potential role in B cell development. To enable these studies, we conducted RNA-sequencing on ribosomal RNA depleted, polyA neutral total (and nuclear extracted) RNA from two primary cell lines genetically arrested at distinct stages of early B cell development. We developed a user/biologist friendly workflow (that accepts standard RNA-seq output data) that identifies high confidence lncRNAs from the thousands of annotated as well as unannotated transcripts identified by RNA-seq. This 'TRUElncRNA' workflow identified ~190 novel lncRNAs from the ~38,000, 10% of which are highly (FPKM > 5) and differentially expressed. In efforts to ensure we are truly investigating lncRNAs that do not code for proteins shorter than 100 amino acids, we have restricted our functional assays to nuclear retained lncRNAs. To this end, we have employed cytological and molecular based analyses to determine the sub-cellular distribution of our highly expressed high confidence lncRNAs. Here we show the sub-cellular distribution patterns of our panel of high confidence lncRNAs in their native cellular states.

P1161**Board Number: B494****Cell-Free Packaging of MicroRNA into Exosomes Reveals Y-box Protein I as a Critical Sorting Factor.**M.J. Shurtleff¹, K.V. Karfilis², R.W. Schekman³;¹Department of Plant and Microbial Biology, University of California at Berkeley, Berkeley, CA, ²Institute of Molecular Biology, University of Oregon, Eugene, OR, ³Department of Molecular and Cellular Biology, University of California at Berkeley, Berkeley, CA

Exosomes are 30-100 nm vesicles that are released to the cell exterior. While numerous studies report the enrichment of select microRNAs (miRNA) in exosomes, little is known about how exosomal miRNAs are selectively packaged. Mechanistic studies in exosome biology are typically conducted with cells in culture, usually involving knockdown and over-expression of putative sorting or targeting proteins. Unfortunately, this approach suffers from possible indirect effects on exosome biogenesis or packaging. In this study, we sought to identify and functionally validate exosomal microRNA sorting components by investigating miRNA packaging into exosomes directly in a cell-free biochemical reaction.

To identify selectively packaged miRNAs, we purified exosomes from HEK293T conditioned media using a three-step purification strategy. Illumina-based small RNA sequencing revealed several miRNAs that are highly enriched in exosomes compared to cells. Next, we developed a cell-free reaction to detect the selective incorporation of an exosomal microRNA. Cytosol and crude membranes prepared from HEK293T cells were mixed with synthetic miRNA (miR-223) and ATP and incubated at 30°C. MiR-223, but not a cellular miRNA (miR-190), was incorporated into an RNase-protected membrane in a detergent sensitive manner. Protection was dependent on cytosolic proteins, membranes, ATP and incubation at physiologic temperature. To identify proteins that are directly involved in packaging miR-223, we combined *in vitro* packaging with proteomics. Y-box protein I (YBX1) co-precipitated with biotinylated miR-223 pulled-down from a complete cell-free reaction. We next generated a YBX1 knockout cell line (Δ YBX1) by CRISPR/Cas9 genome editing and found that cytosol from Δ YBX1 cells did not support miR-223 packaging *in vitro* but that packaging was restored with cytosol from Δ YBX1 cells transfected with YBX1. Δ YBX1 cells were only partially defective in miR-223 secretion due to compensatory overexpression of a germ-line restricted YBX1 paralog (YBX2). However, secretion of miR-223 was further diminished when YBX2 was depleted in Δ YBX1 cells. Our results show that efficient packaging of exosomal miRNA *in vitro* and *in vivo* requires Y-box proteins with YBX1 as the dominant sorting factor in HEK293T cells.

P1162**Board Number: B495****Generation of in vivo circular and snRNA reporters.**C.A. Schmidt¹, A.G. Matera^{1,2};¹Curriculum in Genetics and Molecular Biology, University of North Carolina, Chapel Hill, NC,²Departments of Biology and Genetics, Integrative Program for Biological and Genome Sciences, Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC

Previous attempts to study RNA in the cell have been dependent upon fluorescently tagged RNA binding proteins. The problem with this approach is that nonspecific binding can occur, and binding does not enhance the fluorescence of the protein. An alternative strategy is to utilize RNA aptamers that specifically enhance the fluorescence of a small molecule, such as the Spinach2-DFHBI system.

We have used Spinach2 to generate specific RNA reporters. First, we have created a series of snRNA reporters by replacing the majority of the coding sequence of Sm-class snRNAs with Spinach2 while retaining the Sm site and 3' stem loop. We plan to make transgenic flies so that we can answer fundamental questions about snRNA transport in *Drosophila melanogaster* development.

Second, we aim to study the biogenesis of tRNA intronic circular (tric)RNAs, which are a class of abundant non-coding RNAs that arise from the circularization of tRNA introns. We discovered tricRNAs using bioinformatic analysis of chimeric RNA-seq reads. Strikingly, we see a high degree of tricRNA conservation among Drosophilids, suggesting a potential function. To investigate the biogenesis of tricRNAs, we have utilized the Spinach2 fluorescent RNA system by replacing a tRNA intron with the Spinach2 gene. Circularized Spinach2 is fluorescent, and we can detect the difference in linear vs circular Spinach2 by a polyacrylamide gel assay. Experiments aimed at testing factors necessary for circularization using *Drosophila melanogaster* mutants will be presented.

P1163**Board Number: B500****Investigating the role of Npl3 in maintaining yeast telomeres.**J.Y. Lee-Soety¹, J.K. Jones¹, A. Ito¹, S. Spencer¹, M. Tremoglie¹;¹Biology, Saint Joseph's University, Philadelphia, PA

Linear chromosomes in eukaryotic cells end with telomere structures. In addition to safeguarding the integrity of the genome, the telomeres serve to protect the cell from perceiving the chromosome end as DNA damage and prevent cell cycle arrest. A variety of proteins have been shown to maintain the telomere. Telomerase, in particular, elongates the chromosome end and counteracts the end replication problem. Human somatic cells or mutant yeast cells that no longer express telomerase have progressively shortened telomeres which eventually leads to cell cycle arrest, also known as replicative senescence. Recently, several studies have shown that non-coding RNA is synthesized from the long-

thought transcriptionally silent telomeric region. These telomeric noncoding RNA (TERRA) molecules have roles in coordinating telomere-binding proteins. Our lab has found that yeast Npl3, an hnRNP-related protein that processes RNA, is involved in telomere maintenance since cells with mutations in both telomerase and Npl3 (*tlc1 npl3*) senesced much faster than cells with only the telomerase mutation (*tlc1*). In *tlc1 npl3* mutants, the levels of TERRA are also significantly increased compared to *tlc1* cells. This prompted us to investigate whether Npl3 participates in regulating chromatin structure at the telomeres to facilitate TERRA transcription or in promoting efficient TERRA elongation. It does not appear that deleting *HST1* (encodes NAD⁺-dependent histone deacetylase and involved in telomere maintenance) accelerates the senescence of *tlc1* or *tlc1 npl3* cells; this suggests that Npl3 may not work such histone modification molecule to maintain the “closed” chromatin at the telomeres. However, it is possible that Npl3 functions with Tho2 (required for transcription elongation) since *npl3 tho2* double mutants are non-viable. It will be very interesting to study this interaction at the telomeric region. Understanding the maintenance of telomeres has significant applications in cell aging and cancer.

P1164

Board Number: B501

Direct RNA binding stabilizes the histone methyltransferase SUV39H1 at pericentric heterochromatin.

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Histone modifying enzymes regulate eukaryotic chromatin compaction and transcription, but how their interactions with chromatin are established and maintained is poorly understood. The conserved histone H3 methyltransferase Clr4/Su(var)3-9/SUV39H1 creates a platform for heterochromatin assembly by methylating histone H3 on lysine 9, providing a specific binding site for chromodomain-containing proteins including SUV39H1 itself and the essential Heterochromatin Protein 1 (HP1). In humans, constitutive heterochromatin formed by SUV39H1 is important for silencing transcription from the repetitive DNA sequences surrounding centromeres. This pericentric heterochromatin is the site for sister chromatid cohesion in mitosis, which is necessary for proper chromosome alignment and segregation. Defective pericentric heterochromatin and aberrant transcription of pericentric repeats is associated with genomic instability and cancer. In fission yeast, some transcription of pericentric RNA is necessary for recruiting heterochromatin proteins to pericentric chromatin, but it is unclear how human cells recruit and maintain heterochromatin proteins at pericentric regions. Using a method to specifically label RNA in human cells, we find that RNA is stably associated with the pericentric heterochromatin of human mitotic chromosomes, and colocalizes with SUV39H1. Purified SUV39H1 directly binds RNA in vitro through its chromodomain, in a manner that is not sequence specific but dependent on RNA length. Immunoprecipitation of tagged SUV39H1 from crosslinked cells reveals an in vivo interaction between SUV39H1 and repetitive centromeric RNA, which can be disrupted by a point mutation in SUV39H1 that reduces its direct binding to RNA. This mutation in SUV39H1 that disrupts RNA binding, or specific digestion of RNA by RNase, leads to loss of SUV39H1 localization from mitotic chromosomes.

We propose a model in which RNA associated with pericentric heterochromatin directly binds to SUV39H1, ensuring its stable association with chromatin and robust silencing of this heterochromatin domain.

P1165

Board Number: B502

Bovine microRNAs are bioavailable and affect gene expression in humans and mice.

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Claims that mammals absorb biologically effective amounts of plant microRNAs are highly controversial and have largely been dismissed by the scientific community. Here, we tested the hypotheses that (1) humans absorb biologically meaningful microRNAs from nutritionally relevant doses of milk, (2) milk-borne microRNAs regulate human gene expression, and (3) mammals cannot compensate for dietary microRNAs depletion by endogenous microRNA synthesis. Studies in colon carcinoma caco-2 cells suggest that human intestinal cells transport fluorophore-labeled bovine milk exosomes using a carrier-mediated process that exhibits saturation kinetics and depends on surface proteins in exosomes and cells. When healthy adults consumed nutritionally relevant doses of cow's milk, postprandial concentration time curves suggest that meaningful amounts of microRNA-29b (miR-29b) and microRNA-200c (miR-200c) were absorbed; plasma concentrations of miR-1 did not change (negative control). The expression of RUNX2, a known target of miR-29b, increased by 31% in blood mononuclear cells following milk consumption compared with baseline. When milk exosomes were added to cell culture media, mimicking postprandial concentrations of miR-29b and miR200c, reporter gene activities significantly decreased by 44% and 17%, respectively, compared with vehicle controls in HEK-293 human kidney cells. Our bioinformatics analyses predict that 175 microRNAs in bovine milk target about 10,000 human genes. When C57BL/6J mice were fed a milk microRNA-depleted diet for four weeks, plasma miR-29b levels were significantly decreased by 61% compared with microRNA-sufficient controls, i.e., endogenous synthesis did not compensate for dietary depletion. When humans consumed a broccoli sprout meal, no postprandial increases were observed for plant-specific miR-167a and brassica-specific miR-824 in plasma. We conclude that exosomes from bovine milk are bioavailable and elicit changes in gene expression in humans. [Supported by the National Institute of Food and Agriculture (multistate grant W3002) and the NIH (R01 DK063945, R01 DK077816, and 1P20GM104320)].

The Nuclear Envelope and Nuclear Pore Complexes

P1166

Board Number: B504

Nuclear displacement by centrifugal force reveals distinct LINC complexes engage microtubules and actin filaments to actively maintain nuclear position.

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Nuclei reside at specific sites that are characteristic of cell type. Except in cases where nuclei are actively moved, it is unclear whether nuclear positioning is an active process and whether the specific position is important for cell function. To address these issues, we developed a novel technique to displace nuclei by subjecting adherent cells to centrifugal force. Nuclear displacement was proportional to force from 1,000-20,000 x g and the centrosome, Golgi, ER or mitochondria were not displaced. If wounded fibroblast monolayers were placed orthogonal to the centrifugal force, nuclei in cells at the wound edge were displaced to the rear on one side, but towards the front on the other. After centrifugation, nuclei returned to the center from both positions, but at different rates and by different cytoskeletal mechanisms: front-to-center movement was inhibited by actin and myosin II inhibitors, whereas rear-to-center movement was inhibited by microtubule and dynein inhibitors and dynein heavy chain knockdown. Using siRNA to deplete LINC complex components revealed that nesprin-2G was required for both movements, SUN1 for the microtubule-dependent re-centering and SUN2 for actin-dependent re-centering. Using small fragments of nesprin-2G to rescue nesprin-2G depleted cells showed that distinct regions of nesprin-2G rescued the microtubule and actin-dependent movements. Our results show positioning of apparently stationary nuclei is an active process and suggest that a single nesprin can form different LINC complexes to engage different cytoskeletal elements to maintain nuclear position.

P1167

Board Number: B505

A novel nuclear re-sizing activity: cPKC is a regulator of nuclear size in Xenopus development.

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Cell and nuclear sizes are tightly regulated amongst species, cell types, developmental stages and disease states. From fertilization of the *Xenopus laevis* egg to the post-gastrulation embryo there is an 8-fold reduction in nuclear surface area. These drastic nuclear size changes occur without changes in DNA content per cell, suggesting nuclear size is regulated by yet unknown cytoplasmic factors. In order to discover developmentally regulated proteins that control nuclear size during *X. laevis* embryogenesis,

we developed an assay whereby large nuclei assembled in egg extract shrink in the presence of late embryo extract (stage 11.5-12). Selective inhibition of protein kinase C (PKC) with chelerythrine blocked shrinking. Furthermore, by depleting calcium and diacylglycerol and by cPKC-specific antibody inhibition, we identified conventional PKC isozymes as being responsible for this activity. Importantly, we find that this PKC-dependent nuclear shrinking is conserved *in vivo*. Treating live embryos with chelerythrine or PMA (a PKC activator) led to increased and decreased nuclear size, respectively during interphase. Interestingly, while PKC protein levels do significantly change during early development, increased PKC activation and translocation to the nucleus were observed in late embryos, concomitant with reduced association of nuclear lamins with the nuclear envelope. Moreover, preliminary studies indicate that the cPKC-dependent nuclear re-sizing activity is conserved outside of development in *X. laevis* somatic cells and mammalian tissue culture cells. We propose a model of steady-state nuclear size regulation whereby nuclear expansion is balanced by an active cPKC-dependent mechanism that reduces nuclear size. Future mapping of PKC phosphorylation sites on nuclear lamins and subsequent phospho-mutant identification of the site that contributes to reducing nuclear size during developmental progression will allow us to directly alter nuclear size and characterize the functional impacts of nuclear size.

P1168

Board Number: B506

LINC Complexes Support Hair Follicle Integrity and Intercellular Adhesion.

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Keratinocytes of the mammalian epidermis and its appendages, including the hair follicle, require robust cell-cell and cell-extracellular matrix adhesions to withstand external mechanical stress. Interestingly, cadherin-based cell-cell adhesion between epithelial cells can alter nuclear positioning, suggesting a link between intercellular junctions and the nucleus. An interconnected cytoskeletal network linking cell-cell adhesions to cell-extracellular matrix adhesions may be integrated at the nucleus through nuclear envelope-spanning LINC complexes. The LINC complex mechanically couples the nuclear interior to the cytoplasmic cytoskeleton to support nuclear migration, positioning, and anchorage in various mammalian tissues, but these functions have not been extensively studied in the epidermis to date. Here, we have identified an unappreciated role for the LINC complex component SUN2 in the maintenance of intercellular adhesion between mammalian epidermal keratinocytes in the murine hair follicle. Mice deficient for SUN2 display defects in follicle structure and integrity during the first hair cycle, including excessive follicular bending and hair shaft breakage, leading to transient alopecia that resolves by the second hair cycle. Ultrastructural analysis revealed alterations in desmosome density between *Sun2*^{-/-} follicular keratinocytes, as well as irregular desmosome morphology and drastic intercellular gaps between cells. While primary keratinocytes derived from *Sun2*^{-/-} mice formed apparently normal desmosomal and cadherin-based adhesions *in vitro*, adhesive monolayers of *Sun2*^{-/-} keratinocytes fragmented significantly more than WT cells when mechanically challenged, indicating

that the resulting adhesions were functionally defective. Further, *Sun2*^{-/-} cells exhibited defective nuclear positioning in response to adhesion induction, with excessive nuclear movement toward intercellular adhesions. A compensatory increase in SUN1 expression in the epidermis of *Sun2*^{-/-} mice may account for the restoration of hair follicle structure during the second hair cycle by reestablishing LINC complex function. Overall, our results support a requirement for SUN2-based connections between the nucleus and cytoskeleton in the formation, maturation, or maintenance of intercellular adhesions both *in vivo* and *in vitro*. These results implicate LINC complexes, and therefore nuclear-cytoskeletal interactions, in the maintenance of cell-cell adhesion integrity, and the overall mechanical integrity of the epidermis.

P1169

Board Number: B507

Quality control of inner nuclear membrane proteins by the Asi complex.

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Misfolded proteins in the endoplasmic reticulum (ER) are eliminated by a quality control system called ER-associated protein degradation (or ERAD). This pathway also mediates the turnover of some folded ER proteins, thereby affecting other ER-related functions such as sterol homeostasis. However, how misfolded membrane proteins in the inner nuclear membrane (INM), a specialized ER subdomain with restricted access, are degraded is not known. Here, a quantitative proteomics approach led to the identification of a novel ERAD branch required for protein quality control in the INM. We show that this branch is defined by the integral membrane proteins Asi1, Asi2 and Asi3 (which assemble into the Asi complex at the INM) and requires the Cdc48 ATPase. Among the substrates of the Asi complex are both misfolded proteins and functional regulators of sterol biosynthesis, a feature common to all ERAD branches. Our findings highlight that the quality control of membrane proteins is spatially segregated between INM and the rest of the ER membrane.

P1170

Board Number: B508

Investigating repair of DNA double-strand breaks in live *S. pombe* cells using a lacO/lacI-GFP system to monitor DSB resection, position, and dynamics.

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A cell must accurately correct DNA damage to maintain its genome. One pathway used to repair DNA double-strand breaks (DSBs) is homologous recombination (HR). In HR, the broken strand of DNA is

paired with a homologous donor strand (usually the sister chromatid in G2) that is then used as a template for repair. A key, early step in HR is nucleolytic degradation of the 5' ends flanking the DSB, a process termed resection. Resection commits a DSB to repair by the HR pathway and facilitates pairing with the homologous donor. Much is known about the molecular details of HR, including its regulation throughout the cell cycle and many of the enzymes that mediate the various steps of repair. However, less is understood about how HR (and more broadly, all types of DNA repair) is influenced by its cellular context: the densely packed and dynamically organized nucleus.

To investigate the role of sub-nuclear compartmentalization in DSB repair by HR, we developed a microscopy-based assay to observe the initial steps of repair in live *S. pombe* cells. Our assay uses a rapid induction system to generate a single, site-specific DSB that is directly adjacent to a lac operator (lacO) array. Binding of lacI-GFP to the lacO array allows us to track the position of the DSB during the course of repair. After DSB induction, loss of dsDNA sequence at the lacO array flanking the DSB due to resection provides a means to monitor various properties that influence the resection rate.

Unexpectedly, our data have revealed that the rate of resection is quite variable within a population of live cells in a manner independent from the cell cycle. We are currently addressing the molecular basis for this heterogeneity by visualizing repair proteins (such as Rad52-mCherry) and assaying strains deficient in various repair proteins. We are also investigating the role of DSB position, especially tethering at the nuclear periphery, in regulating resection.

P1171

Board Number: B509

Surveillance of nuclear pore complex assembly by the ESCRT machinery.

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The assembly of Nuclear Pore Complexes (NPCs) during interphase is thought to occur through the stepwise recruitment of the ~30 nucleoporins (nups) to the nuclear envelope, which occurs concomitantly with the fusion of the inner and outer nuclear membranes. Given that a single defective NPC could compromise the barrier function of the nuclear envelope, it is essential to understand how NPC quality is ensured. Working in budding yeast, we will show evidence for a surveillance mechanism that oversees NPC assembly. Our data are consistent with a model in which surveillance is achieved through the recognition of early NPC assembly intermediates by integral inner nuclear membrane proteins of the Lap2, emerin, MAN1 family, Heh1 and Heh2. When NPC assembly is compromised, Heh2 recruits the Endosomal Sorting Required for Transport (ESCRT) –III subunit, which, along with the AAA-ATPase Vps4, acts to potentially clear and target the degradation of misassembled nups. Under conditions in which surveillance is compromised, defective NPCs accumulate in a compartment that we term the SINC for Storage of Improperly assembled Nuclear pore Complexes. Consistent with the idea that the SINC is a repository for malfunctioning NPCs, it is most prevalent in 'old' mother cells and is almost never transmitted to daughter cells through mitotic divisions. Thus, there are several mechanisms that ensure NPC quality to support proper nuclear compartmentalization. Moreover, our

data highlight an exciting functional relationship between two ancient membrane-bending/associated machineries.

P1172

Board Number: B510

Three-dimensional Super-resolution Mapping the Selective FG-Barrier in Nuclear Pore Complex.

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The nuclear pore complexes (NPCs) selectively mediate the bidirectional trafficking of macromolecules between the cytoplasm and the nucleus in eukaryotic cells. The selective barrier formed by natively unfolded phenylalanine-glycine (FG) nucleoporins (Nups) inside the NPC allows for passive and facilitated transport through the NPC. However, the mechanism of formation and spatial distribution of FG-barrier in the NPC remains unresolved. By single-point edge-excitation sub-diffraction (SPEED) microscopy, we have used various fluorescent transport receptors (TRs) and FG segments as probes to determine the structure of FG-barrier in three dimensions in the native NPC. The three-dimensional spatial locations of probe-based FG repeats for the first time provide the native status of FG barrier in the NPC. Moreover, the competitions among various TRs and between TR and FG segments while binding FG Nups were systematically explored. Two major transport receptors Imp β 1 and CRM1, possessing dominant binding FG Nups over the other transport receptors, surprisingly have distinct transport routes without competition through the native NPC.

P1173

Board Number: B511

SINC, a secreted effector of *Chlamydia psittaci*, targets emerlin and the nuclear lamina of infected cells and uninfected neighbors.

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The chlamydial type III secretion system injects effector proteins into the host cell cytosol to facilitate bacterial growth and pathogenesis. We characterized SINC, a new effector produced by the avian and human pathogen, *Chlamydia psittaci*. SINC is syntenic with CT694 of *Chlamydia trachomatis* and likewise expressed late in development. However SINC uniquely targets the nuclear envelope (NE) of both *C. psittaci*-infected and uninfected neighboring cells. Digitonin-permeabilization studies of infected or SINC-GFP-transfected HeLa cells suggest SINC targets the inner nuclear membrane (INM). Candidate

partners were identified by proximity to biotin ligase-fused SINC (BirA-SINC) in HeLa cells and mass spectrometry (BioID). Among the most abundant peptides were fragments of INM proteins MAN1 and emerin, and of the nuclear pore complex protein, ELYS, suggesting candidate interacting partners at the NE. SINC-GFP association with the native LEM-domain protein emerin of the nuclear lamina was confirmed by GFP pull-down. SINC localization at the NE was blocked by importazole, confirming SINC import into the nucleus. Our findings identify SINC as a novel effector that is transported to the NE of infected and neighboring uninfected cells where it targets LEM domain proteins of the nuclear lamina. This association suggests that *C. psittaci*, an aggressive pathogen, has the capacity to modulate host cell nuclear functions, from chromatin organization to signaling and cytoskeletal regulation, both endogenously and exogenously.

P1174

Board Number: B512

Probing transmembrane protein distribution along the nuclear envelope using super-resolution microscopy.

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The double membrane nuclear envelope (NE) serves as a physical barrier between the cytoplasm and the nucleoplasm in eukaryotic cells. Transmembrane (TM) proteins are synthesized outside the nucleus on the endoplasmic reticulum (ER) and are then transported from the outer nuclear membrane (ONM) to the inner nuclear membrane (INM). Electron microscopy has been the most common approach to determine the distribution of TM proteins along the NE. However, this *in vitro* method requires the time consuming process of either chemical-fixation or frozen cells. Here, we exploit the super-resolution capabilities of the recently developed single-point edge-excitation sub-diffraction (SPEED) microscopy technique to study the distribution of TM proteins on the ONM and INM of the NE *in vivo*. This method allows us to quickly obtain distribution data of TM proteins along the NE in living cells.

P1175

Board Number: B513

Crystal structure of Nup62·58·54 nucleoporin complex.

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Nucleoporins (Nups) are the building blocks of a highly sophisticated translocation machinery in the cell called the Nuclear Pore Complex (NPC), which allows the controlled exchange of macromolecules across the nuclear envelope (NE). Many Nups interact tightly to form subcomplexes that arrange in octahedral symmetry to impart a rigid NPC scaffold. The Nup62·58·54 subcomplex is located near the central NPC translocation channel. It comprises three subunits, Nup62, Nup58 and Nup54, all predicted to contain

coiled coil (CC) domains. The complex is essential, highly conserved in evolution, a major anchor site for NPC barrier-forming FG domains and attached to the NPC via Nup93 (Nic96 in *S.cerevisiae*). The molecular structure of Nup62·58·54 subcomplex is hotly debated, because attempts to crystallize a complete complex have so far failed and only fragments with apparently non-physiological oligomerization propensity and stoichiometry have been crystallized instead (see Science 2007, 315: 1729; Cell 2011, 147:590; PNAS 2013, 110: 5858 and MBoC 2014, 25:1484 for an opposing perspective). On the basis of the fragment structures, it had been proposed that the subunits of Nup62·58·54 complex would slide against each and thereby dilate or narrow the central NPC channel. In order to crystallize a complete xNup62·58·54 complex, we mapped its domain boundaries and reached a stable, hetero-trimeric complex that can also incorporate efficiently into the NEs of *in vitro* assembled nuclei, suggesting a biologically relevant complex. In order stabilize the flexible parts and hinge regions of the complex that interfered with its crystallization, we screened many single-domain antibodies (Nanobodies, Nbs), and found a few that bound the trimeric state and a single one that conferred crystallizability. We also identified the Nup93 binding sites on Nup62·58·54 complex. Crystals obtained from the Nb-bound Nup62·58·54 complex diffracted to 3.2Å. The Nup62·58·54 complex structure revealed a very extensive hetero-trimerisation interface including two hetero-trimeric coiled coil regions that are connected by a sharp kink and pack against each other through another extensive hydrophobic interface. This structure of the biologically relevant Nup62·58·54 complex rules out any sliding between the subunits. The structure includes a number of striking features, whose functional significance is currently being tested.

P1176

Board Number: B514

Determination of the three-dimensional organization of chromatin by modelling-supported selective chromosomal interaction capture (T2C).

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The dynamic three-dimensional chromatin architecture of genomes and the obvious co-evolutionary connection to its function – the storage and expression of genetic information – is still, after ~170 years, a central question of current research. With a systems genomics approach using a novel selective high-throughput chromosomal interaction capture (T2C) technique together with quantitative polymer simulations and scaling analysis of genomic structures and the DNA sequence, we determined the architecture of genomes with unprecedented molecular resolution and dynamic range from single base pair entire chromosomes: for several genetic loci of different species, cell type, and functional states we find a chromatin quasi-fibre exists with 5 ± 1 nucleosome per 11 nm, which folds into 40-100 kbp loops forming aggregates/rosettes which are connected by a ~50 kbp chromatin linker. Polymer simulations using Monte Carlo and Brownian dynamics approaches confirm T2C results and allow to predict and explain additional experimental findings. This agrees also with novel dynamics information from

fluorescence correlation spectroscopy (FCS) analysis of chromatin relaxations *in vivo* (see abstract M. Wachsmuth & T. A. Knoch, Dynamic and structural properties of interphase chromatin mapped *in vivo* with fluorescence correlation spectroscopy and quantitative modelling). Beyond, we find a fine-structured multi-scaling behaviour of both the architecture and the DNA sequence which shows for the first time, that genome architecture and DNA sequence organization are directly linked – again in detail on the base pair level. Hence, we determined the three-dimensional organization and dynamics for the first time in a consistent system genomics manner from several angles which are all in agreement as well as additionally also with the heuristics of the research of the last 170 years. Consequently, T2C allows to reach an optimal combination of resolution, interaction frequency range, multi-plexing, and an unseen signal-to-noise ratio at molecular resolution and hence at the level of the “genomic” uncertainty principle and statistical mechanics, this opens the door to architectural sequencing of genomes and thus a detailed understanding of the genome with fundamental new insights with perspectives for diagnosis and treatment.

P1177

Board Number: B515

ES cell dependence on NUP133 for normal nuclear pore number and density.

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Our previous studies on the mouse mutant, *mermaid* (*merm*), found that a null allele of *Nup133* disrupts differentiation of neural and likely other embryonic lineages (Lupu *et al.*, 2008). *During neural differentiation, NUP133-deficient embryonic stem cells (ESCs) recapitulate aspects of the merm embryo phenotype, including impairment in production of terminally differentiated neurons.*

Immunofluorescence to nucleoporins (NUPs) in *merm* ESCs and neural tubes demonstrated that, despite the absence of NUP133, nuclear pore complexes (NPCs) assemble in the nuclear envelope.

NUP133 is a component of the NUP107-160 (or Y) complex, a conserved structural unit of the NPC. Analyses implicate the Y complex as a participant in multiple cellular processes, including NPC assembly, macromolecular transport, and cell cycle regulation. How the *merm* phenotype results from disruption of these functions remains unknown. To elucidate the requisite roles of NUP133, we have examined nuclear pore number and density in wild type and *merm* ESCs.

To quantify NPC number and density we applied structured illumination super-resolution microscopy (Delta Vision OMX) to acquire images of WT, *Nup133*^{+/-} (Het), and *Nup133*^{-/-} (*merm*) ESC nuclei stained with antibodies to TPR, a nuclear basket component. Using Imaris software (Bitplane) we measured total NPC number and nuclear surface area, enabling an estimate of NPC density. We found comparable numbers in WT vs. Het ESCs but a reduction of number and density of TPR-labeled NPCs in 3 independent *merm* ESC lines.

To determine whether these data reflect a general deficiency in NPC assembly, we examined WT, Het and *merm* ESCs using mAb414, which recognizes a group of FG-repeat-containing NUPs. On WT cells, both antibodies yield similar estimates of NPC number. However, mAb414 detects more pores than anti-TPR in *merm* ESCs, suggesting the presence of incomplete NPCs in the mutant cells. To further evaluate these results, we are quantifying NPCs using antibodies to additional NUPs.

According to current models, two distinct mechanisms direct NPC assembly. One acts post-mitotically, the other during interphase, and each posits different requirements for NUP133. Preliminary evaluation of WT and *merm* ES cells, in G1 vs. G2, suggests that loss of NUP133 affects both pathways.

In addition, we are using *merm* ESCs stably expressing WT or mutant forms of GFP-mNUP133, and will present rescue experiments to assess the extent to which neural differentiation depends on NPC number and density in the nuclear envelope of a progenitor cell.

P1178

Board Number: B516

The role of SUN proteins in nuclear envelope spacing of force-bearing cells.

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The nuclear envelope is made up of two membranes, the inner and outer nuclear membranes, separated by a uniform distance of 40-50 nm, known as the perinuclear space. The mechanism by which even spacing of the two nuclear membranes is maintained has been poorly understood. Sad1p-UNC-84 (SUN) proteins, which interact with Klarsicht, ANC-1, and SYNE homology (KASH) proteins to connect the nucleoskeleton to the cytoskeleton, have been implicated as molecular rulers that set the nuclear envelope space. This hypothesis was based on the observation that HeLa cells depleted of SUN proteins show blebbing of the outer nuclear membrane away from the inner nuclear membrane. To test the hypothesis that the *C. elegans* SUN protein UNC-84 spans and regulates the perinuclear space, we examined the morphology of the nuclear envelope by electron microscopy in *unc-84(null)* mutant animals. In most *unc-84(null)* mutant tissues, nuclear envelope morphology was normal. Therefore, SUN proteins are not necessary to regulate nuclear envelope spacing. However, we did observe extreme separation of the outer nuclear membrane from the inner nuclear membrane in *unc-84(null)* L1 body wall muscle nuclei. These mutant animals also displayed irregular locomotion in liquid and perhaps serve as a model for human laminopathies due to defects in LINC complex proteins that have reduced muscle function. Our data suggest a correlation between nuclear envelope morphology and muscle function. Surprisingly, UNC-84 protein with a 300 amino acid deletion in the luminal domain was able to form functional nuclear envelope bridges, but did not have a noticeable effect on nuclear envelope spacing. We propose that SUN proteins are only required to maintain the nuclear envelope space in cells that are under strain. Rather than dictating the perinuclear space, SUN proteins have appeared to evolve to reach across it.

P1179**Board Number: B517****Karyopherin-centric control of nuclear pores based on multivalent binding with FG Nucleoporins.**L.E. Kapinos¹, R.L. Schoch¹, K.D. Schleicher¹, R.S. Wagner¹, R.Y. Lim¹;¹Biozentrum, University of Basel, Basel, Switzerland

Intrinsically disordered Phe-Gly nucleoporins (FG Nups) within nuclear pore complexes (NPCs) exert multivalent interactions with transport receptors (Karyopherins or Kaps) that orchestrate nucleocytoplasmic transport. Current FG-centric views reason that selective Kap translocation is promoted by alterations in the barrier-like FG Nup conformations. However, the strong binding of Kaps with the FG Nups due to avidity contradicts rapid Kap translocation *in vivo*, thereby underscoring how mechanistic and kinetic views of NPC functionality remain at odds. Here, I will discuss our recent efforts to understand how multivalent Kap-FG Nup binding impacts on transport selectivity and speed. This includes (i) studying the crosstalk between equilibrium affinity and kinetic rates of Karyopherin β 1 (Kap β 1) binding with conformational changes in the FG Nups *in situ*; and, (ii) assaying Kap-facilitated transport when binding FG Nups *in vitro*. A characteristic feature of the FG Nups is their ability to accommodate large numbers of Kap β 1 molecules at physiological concentrations by conformational changes. However, whereas most bound Kap β 1 molecules become long-lived constituents inside a FG Nup layer due to strong binding avidity, Kap β 1 molecules located at the layer periphery are weakly bound and dominate fast transport kinetics due to limited binding with the pre-occupied FG Nups. Hence, the number of free FG-repeats in a FG Nup layer, and the strength of Kap β 1 binding are directly correlated to the amount of Kap β 1 in solution. As proof-of-principle, we find that varying Kap β 1 concentration can control Kap β 1-facilitated motion on a FG Nup layer. This evokes differential behavior ranging from highly constrained to two-dimensional diffusion (i.e., Reduction of dimensionality) at physiological Kap β 1 concentrations. In contrast to FG-centric views, I will discuss how our findings show support for an emergent Kap-centric mechanism that may underlie barrier functionality and selective transport control in NPCs.

1. R.L. Schoch, L.E. Kapinos, and R.Y.H. Lim, Nuclear transport receptor binding avidity triggers a self-healing collapse transition in FG-nucleoporin molecular brushes. *PNAS* 109 16911 (2012)
2. L.E. Kapinos, R.L. Schoch, R.S. Wagner, K.D. Schleicher and R.Y.H. Lim, Karyopherin-centric control of nuclear pores based on molecular occupancy and kinetic analysis of multivalent binding with FG-Nucleoporins. *Biophys. J.*, 106 1751 (2014)
3. K.D. Schleicher, S.L. Dettmer, L.E. Kapinos, S. Pagliara, U.F. Keyser, S. Jeney and R.Y.H. Lim, Selective transport control on molecular velcro made from intrinsically disordered proteins. *Nature Nanotechnology*, 9 525 (2014)

P1180**Board Number: B518****A genetic screen reveals the requirement for mRNA processing genes and the topoisomerase TOP2 in nuclear morphology.**R.A. Meseroll¹, O. Cohen-Fix¹;¹National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), National Institutes of Health, Bethesda, MD

The mechanisms dictating the size and shape of the nucleus in any cell type are poorly understood. The nucleus of the budding yeast *Saccharomyces cerevisiae* is round during interphase, with a crescent-shaped nucleolus located at the nuclear periphery. Previous work from our lab has demonstrated that nuclei in cells with upregulated phospholipid biosynthesis or that have been delayed in mitosis form a single extension or “flare” specifically at the nuclear envelope adjacent to the nucleolus. What are the mechanisms that contribute to nuclear morphology and confine expansion to the nucleolar region? To test this, we conducted a screen of randomly-generated conditional temperature-sensitive mutants to identify mutants that have multiple extensions or other abnormalities that form at sites away from the nucleolus. The screen yielded four mutants with the desired phenotype, encoding for single point mutations in three mRNA processing or splicing genes (*CEG1*, *PRP9*, and *PRP22*) and the topoisomerase *TOP2*. All four genes are essential and at least half of the nuclei in the mutants at the restrictive temperature have multiple extensions at sites other than the nucleolus. In addition to the nuclear morphology defects, some of these mutants also have nucleolar defects, such as collapsed nucleoli or nucleoli that appear detached from the nuclear periphery. It remains unclear whether the phenotypes in the mRNA processing and splicing mutants are due to a response to a global defect in mRNA processing or if improper capping or splicing of a particular message or subset of messages is responsible for the abnormal nuclear shape. Presently, we have focused our efforts on determining the role of *TOP2* in nuclear morphology. Topoisomerases are highly conserved proteins responsible for disentangling topological problems that result from DNA double helix unwinding. We hypothesize that nuclear morphology depends on chromatin structure and that aberrant chromatin conformation in the *top2* mutant leads to the nuclear shape defects we observe. We are currently characterizing nuclear and nucleolar defects, testing topoisomerase activity, and examining chromatin organization in the *top2* mutant to determine *TOP2* function in nuclear morphology.

P1181**Board Number: B519****Quantifying Nucleoporin Stoichiometry Inside Single Nuclear Pore Complexes In Vivo.**L. Mi¹, A. Goryaynov², M. Rexach³, A. Lindquist³, W. Yang⁴;¹Department of Optical Science and Engineering, Fudan University, Shanghai, China, ²Department of Cell Biology, Yale University School of Medicine, New Haven, CT, ³Department of Cell and Developmental Biology, University of California, Santa Cruz, Santa Cruz, CA, ⁴Biology, Temple University, Philadelphia, PA

The nuclear pore complex (NPC) is one of the largest supramolecular structures in eukaryotic cells. Its octagonal ring-scaffold perforates the nuclear envelope and houses a molecular sortase that regulates nucleocytoplasmic transport. It is composed of ~30 different nucleoporins (Nups), estimated at 8, 16 or 32 copies per NPC, although this has not been confirmed due to the inherent difficulty of counting proteins within supramolecular complexes. Here we used single-molecule SPEED microscopy to directly count the copy-number of twenty-four different Nups within the NPC of live yeast, and found significant deviations from previous estimates. Each NPC contained a maximum of 16 copies of Nsp1 and Nic96, rather than 32 as previously estimated; 16 copies of thirteen additional Nups; 8 copies of four Nups; and 10-15 copies of five Nups. This in situ molecular-counting technology can help resolve the architecture of NPCs and other supramolecular structures in living cells.

P1182**Board Number: B520****Nuclear size scaling during *Xenopus* early development contributes to the regulation of midblastula transition timing.**P. Jevtic¹, D. Levy¹;¹Department of Molecular Biology, University of Wyoming, Laramie, WY

Early *Xenopus laevis* embryogenesis is a robust system for investigating mechanisms of developmental timing. The first major developmental transition is the midblastula transition (MBT), when zygotic transcription begins and cell cycles elongate. Fertilization is followed by a series of rapid cell divisions with concomitant reductions in cell size and it has long been recognized that the nuclear-to-cytoplasmic (N/C) volume ratio changes dramatically during early *Xenopus* development. We quantified blastomere and nuclear sizes in *X. laevis* embryos and demonstrate that the N/C volume ratio increases rapidly prior to the MBT. Rather than examining the role of DNA amount in MBT timing, we investigated if MBT timing is regulated by nuclear size and developmental changes in the N/C volume ratio. We manipulated nuclear volume in embryos by microinjecting different nuclear scaling factors, including import proteins, lamins, and reticulons. Using this approach, we first monitored the onset of zygotic gene expression by performing whole-mount in situ hybridization to detect the GS17 transcript. We found that increasing the N/C volume ratio in pre-MBT embryos leads to premature activation of GS17 gene transcription, and

decreasing the N/C volume ratio delays GS17 transcription in MBT embryos. To corroborate our in situ hybridization data, we quantified additional zygotic transcripts (*xnr5-13*, *xnr3*, and *bix1.1*) by qPCR. Stage 6.5 embryos with increased nuclear size showed increased expression of MBT transcripts compared to GFP control-injected embryos, up to seven times greater in some cases. In late stage 8 embryos with decreased nuclear size, we observed less expression of zygotic transcripts compared to controls. Next, we analyzed time-lapse movies of microinjected embryos to test if altering nuclear size in early embryos affects the timing of cellular hallmarks of the MBT. Generally, control GFP-injected embryos cleaved synchronously until the 12th cleavage (the normal MBT), at which time cell cycles lengthened. Embryos with decreased nuclear size cleaved rapidly until, on average, the 14th cell cycle, while embryos with increased nuclear size exhibited longer cell cycles after only the 10th cleavage. Taken together, our data show that nuclear size scaling and changes in the N/C volume ratio that occur during early *Xenopus* embryogenesis act as an additional mechanism in the regulation of MBT timing, demonstrating the functional significance of nuclear size during development.

P1183

Board Number: B521

DNA zip codes induce relocalization to the nuclear periphery and mediate interchromosomal clustering of GAL genes.

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To allow growth in galactose, yeast cells induce a number of genes. Active *GAL* genes in yeast are targeted to the nuclear periphery/nuclear pore complex by cis-acting “DNA zip codes” in their promoters. Genes with identical zip codes cluster together and insertion of zip codes at an ectopic site induces clustering with endogenous *GAL* genes having the same zip code. Clustering of genes is specific; pairs of *GAL* genes that are targeted to the nuclear periphery by different zip codes do not cluster together. The molecular mechanism controlling targeting to the NPC is distinct from the molecular mechanism controlling interchromosomal clustering. Targeting to the nuclear periphery and interaction with functional nuclear pores is a pre-requisite for gene clustering. However, clustering can be maintained in the nucleoplasm and is regulated differently through the cell cycle. Also, although targeting of genes to the NPC is independent of transcription, interchromosomal clustering requires transcription. Finally, although targeting to the NPC promotes stronger expression, removal of a single gene from interchromosomal clusters does not affect the expression of the remaining genes in the cluster. These results suggest that genomes encode elaborate signals that mediate temporally dynamic spatial organization of the genome.

P1184**Board Number: B522****PLK1 functions in nuclear pore complex disassembly.**M.I. Mayr¹, C. Wandke¹, U. Kutay¹;¹Institute of Biochemistry, ETH Zürich, Zürich, Switzerland

Disintegration of the nuclear envelope (NE) is a prerequisite for mitotic spindle assembly in higher eukaryotes. Phosphorylation of nuclear pore complex (NPC) constituents and nuclear membrane proteins is key in initiating diverse steps of nuclear envelope breakdown (NEBD). We have previously shown that cyclin-dependent kinase 1 (CDK1) and NIMA-related kinases (Nek-6 and -7) are crucial for timely nuclear permeabilization in HeLa cells (Laurell et al., Cell, 2011). Reportedly, several nucleoporins (Nups) are phosphorylated on sites matching the consensus for polo-like kinase 1 (PLK1), raising the possibility that PLK1 directly contributes to NE permeabilization during NEBD. In support of this notion, we have observed that PLK1 localizes at the NE during prophase. Specifically, the noncatalytic carboxy terminus of the kinase, the polo-box domain (PBD) is sufficient for targeting to the NE, indicating that NE localization depends on priming phosphorylation. To explore a function of PLK1 in NPC disassembly, we applied a previously developed in vitro system that recapitulates nuclear disassembly on nuclei of semipermeabilized HeLa cells upon addition of mitotic extracts. Chemical inhibition of PLK1 activity as well as immunodepletion of PLK1 significantly delayed the kinetics of NPC disassembly in vitro without affecting CDK1 activity. Add-back of purified wild-type kinase restored timely NE permeabilization. To examine whether PLK1 function during NPC disassembly depends on the PBD we added GST-PBDWT or GST-PBDAA to mitotic extracts to compete with endogenous kinase activity. The addition of GST-PBDWT but not GST-PBDAA significantly delayed NPC disassembly kinetics. To understand the molecular mechanism of how Plk1 phosphorylation affects NPC disassembly, we are currently studying how PLK1-mediated phosphorylation of several candidate nucleoporins affects mitotic NPC disassembly.

P1185**Board Number: B523****Characterization of the interactions between Mps3, Elg1, and Cdc5.**L.M. Antoniaci¹, C. Brown²;¹Science, Marywood Univ, Scranton, PA, ²Science, Marywood University, Scranton, PA

Cdc5 is a polo-like kinase identified in *Saccharomyces cerevisiae*. Cdc5 functions in regulation of many essential cell pathways including Meiosis, Mitosis, and Cytokinesis. A genome wide screen for physical interactors of Cdc5 identified Mps3 as a potential candidate. Our lab has confirmed this interaction using GST pull down and Co-Immunoprecipitation. Mps3 is a nuclear envelope protein involved in several chromosome functions such as sister chromatid cohesion, DNA damage repair, and telomere clustering. Mps3 is hypothesized to be involved in many of these processes through its association with many chromatin associated proteins. One of these chromosome associated proteins is Elg1, which functions in many of the same processes of Mps3. Here we present data that Cdc5 and Elg1 also

physically interact. In addition we investigate the possibility that both Elg1 and Mps3 are substrates of Cdc5.

P1186

Board Number: B524

Characterization of the unique KASH domain protein *Lrmp*.

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KASH domain proteins are key components of the LINC complex (linker of nucleoskeleton and cytoskeleton). The LINC complex is formed by the interaction of SUN (Sad1, Unc-84) domain proteins and KASH (Klarsicht, ANC-1, Syne Homology) domain proteins. The interaction of both protein families builds the bridge between the nuclear lamina and the microtubule and actin network in the cytoplasm. Proteins of the KASH domain family are localized at the outer nuclear membrane, and interact via their C-terminal KASH domain with SUN (Sad1, Unc-84) domain proteins in the perinuclear space. Their cytosolic N-terminus connects with components of the cytoskeleton. In human and mouse, six members of the KASH domain protein family have been described, encompassing Nesprin1-4, KASH5, and the recently identified lymphoid-restricted membrane protein (*Lrmp*). *Lrmp* is localized to the ER, but it can be recruited via its conserved KASH domain to the nuclear envelope by SUN domain proteins. To investigate the physiological function of *Lrmp*, we created a *Lrmp*-deficient mouse strain (*Lrmp*^{-/-}) expressing a *LacZ* reporter gene. Performing a β-galactosidase staining on various tissues derived from *Lrmp*^{WT/-} and *Lrmp*^{-/-} mice, we can trace the expression of *Lrmp* in taste buds of the lingual papillae. Furthermore, we show that *Lrmp* is exclusively expressed in type II taste receptor cells which perceive sweet, umami and bitter flavours. This leads us to the question if *Lrmp* has a pivotal role in taste perception. We compared the taste preference of wild-type and *Lrmp*^{-/-} mice during a two-bottle preference test. However, taste perception in mice lacking *Lrmp* is indistinguishable from the wild-type littermates. We conclude that *Lrmp* is not essential for taste transduction and signalling. In this study, we can show a new expression pattern of *Lrmp*, a unique KASH domain protein, in the taste buds of mice. In future work, we will further investigate the physiological function of *Lrmp* and the LINC complex in taste receptor in conjunction with other putative proteins with which *Lrmp* interacts.

P1187

Board Number: B525

RecQL DNA Helicase Impact on the Nuclear Pore Complex in Aging Cells.

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From the surrounding shell to the inner machinery, nuclear proteins provide the functional plasticity of the nucleus. This study first highlights the association of nuclear pore NDC1 and Werner protein (WRN),

a RecQL helicase responsible for the DNA instability in some Progeria disorders. RecQL helicases are the caretaker of the genome acting to preserve genetic coding and therefore protect cells against aging. Likewise, Nuclear Pore Complexes (NPCs) maintain proper nuclear functionality through the transport of protein and mRNA. In established WRN knockout cell lines, we further demonstrate the interdependence of WRN, LaminB1 and nuclear pore complex nucleoporins. These changes do not completely change the barrier of the nuclear envelope, but do affect the distribution of transport FG Nups and the RAN gradient. Evidence from WRN knockout cell lines demonstrates changes in the processing and nucleolar localization of lamin B1. Furthermore we have found that other RecQL helicases effect the distributions and levels of Nups. We are gaining further proof of this NPC, RecQL helicases and lamin B1 triad.

P1188

Board Number: B526

On The Stoichiometry Of Channel Nucleoporins Nup62, Nup54 And Nup58: A Critical Appraisal.

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Of the 30 nucleoporins (nups) that make up the nuclear pore complex (NPC), three are centrally located: Nup62, Nup54 and Nup58. We recently pieced together the molecular architecture of the NPC transport channel from crystal structures of the binary complexes of Nup62•Nup54 and Nup54•Nup58 (3) and proposed a novel mechanism for how the central channel of the NPC opens and closes by dilation and constriction of mid-plane rings ('ring cycle') (2). Collectively, our crystal structures indicated a stoichiometry of 4:2:1 for Nup62, Nup54 and Nup58, respectively. However, solution studies of the three-protein complex had pointed towards its dynamic nature. In marked contrast, recent solution studies of structured regions of these three proteins suggested that they form a 1:1:1 'triple' complex. To address this ambiguity, we carried out additional solution studies. We found that Nup62, Nup54 and Nup58 do not form a 1:1:1 complex, but instead associate with each other in unstable mixtures of various stoichiometric ratios (1). The polydisperse nature of the ternary complex was demonstrated either by multi-dimensional electrophoretic analysis or by size-exclusion chromatography coupled to multi-angle light scattering. Moreover, probing the ternary complex with wild-type or mutant Nup54 'protomers' yielded data that strongly support the interaction domains of the three channel nups that were established previously by crystallographic methods. Specifically, we show that Nup58 interacts transiently with Nup54 in the assembled ternary complex, which is the basis for its observed heterogeneity. Our data support the 'ring cycle' model of nuclear transport, in which transient interactions between Nup58 and Nup54 would lead to transitions between dilated and constricted states of the transport channel. Specifically the transport of large cargo such as viruses, mRNPs and ribosomal subunits remains poorly understood, even though these processes impact crucial cellular

functions, such as RNA metabolism. To this end, the ring cycle provides a molecular basis for how the NPC can facilitate selective transport of cargo with a size range of 10-50 nm. Besides providing support for the ring cycle model, this study also addresses the widely discussed issue of the stoichiometry of the channel nups, providing a rationale for the different observations reported in the literature.

[1] **Solmaz, SR**, Sharma, A, Blobel, G and Melcak, I. On The Stoichiometry Of Channel Nucleoporins Nup62, Nup54 And Nup58: A Critical Appraisal. Submitted.

[2] **Solmaz, SR**, Blobel, G and Melcak, I. Ring cycle for dilating and constricting the nuclear pore. Proc Natl Acad Sci U S A 2013 110: 5858-5863.

[3] **Solmaz, SR**, Chauhan, R, Blobel, G, and Melcak, I. Molecular architecture of the transport channel of the nuclear pore complex. Cell 2011 147: 590-602.

Endocytic Trafficking 2

P1189

Board Number: B528

Quantitative 4D visualization of clathrin-coated vesicle formation in mammalian cells by correlated light and electron microscopy.

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Clathrin-mediated endocytosis (CME) is a conserved form of selective plasma membrane internalization in eukaryotic cells that plays a fundamental role in numerous physiological and pathological processes. Many components of the multi-protein machinery driving endocytosis have been identified in mammals. However, a comprehensive view of membrane shape transitions during coated vesicle formation is still missing. Clathrin coated vesicle (CCV) budding may occur either through increase in curvature of a pre-assembled coat, or through coat growth, with the curvature of the emerging bud remaining constant. These two models predict different membrane intermediates. Furthermore, for a coated membrane area to change in curvature, the overlying coat must constantly restructure. To distinguish between these models we visualized clathrin-coated pits (CCPs) by correlating fluorescence microscopy and electron tomography in genome-edited mammalian cells expressing fluorescently tagged clathrin and dynamin at physiological levels. We correlated clathrin fluorescence to endocytic sites ranging from flat to highly curved membrane patches and to coated vesicles. We focused on productive endocytic events by analyzing sites on the non-adherent surface of the plasma membrane that were able to recruit cargo. We quantified the curvature and coated surface area of over 200 CCPs and CCVs and found that the curvature of the membrane at the tip of the invagination increases during endocytosis while the area of coated membrane remains constant. These results imply that endocytosis occurs predominantly by inward deformation of flat clathrin lattices. In addition, the population labeled by both clathrin and

dynamin consisted exclusively of deep invaginations that showed membrane constriction at their base, consistent with the known role of dynamin in membrane scission at late stages of CME. To test whether the clathrin coat is remodeled during CME we performed dual color FRAP analysis of single endocytic sites containing clathrin-RFP and dynamin-GFP. Our preliminary results show that clathrin fluorescence largely recovers even in the presence of dynamin suggesting that clathrin is exchanged even at late stages of CME. Taken together, these results suggest that the coat is a dynamic structure that reshapes during endocytosis. Our quantitative 4D description of membrane shape transitions during CME in vivo provides strong evidence for budding via continuous increase in membrane curvature of a constant pre-coated membrane area.

P1190

Board Number: B529

Ultrastructure of the actin cytoskeleton associated with sites of clathrin-mediated endocytosis in dynamin2-depleted HeLa cells.

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Clathrin-mediated endocytosis (CME) plays an essential role in selective uptake of extracellular molecules and plasma membrane components in eukaryotic cells. The formation of an endocytic vesicle is a complex process operated by multiple proteins and the actin cytoskeleton is an important component of the molecular machinery driving endocytic vesicle internalization. Actin cytoskeleton transiently assembles at clathrin-coated pits toward the end of the endocytic cycle, shortly before the clathrin-coated vesicle is pinched off from the plasma membrane by dynamin2. Our understanding of specific roles of the actin cytoskeleton remains incomplete due to lack of detailed ultrastructural information. To get detailed insights into actin cytoskeleton architecture at the endocytic sites, we depleted dynamin2 in HeLa cells by lentiviral shRNA in order to block CME before the vesicle scission step. Platinum replica electron microscopy of unroofed cells revealed a dramatic increase in the fraction of clathrin-coated structures associated with the actin cytoskeleton in most dynamin2-depleted cells, as compared with control cells containing endogenous dynamin2. Actin filaments associated with clathrin-coated structures in dynamin2-depleted cells were organized into branched networks with the shape of long comet tails, in which barbed ends were oriented toward the clathrin-coated structure. These data support the idea that branched actin networks promote invagination of the endocytic bud and elongation of the bud neck by pushing onto the clathrin-coated structure with their barbed ends. This study sets the stage for better understanding of actin-dependent mechanisms of vesicle internalization during CME.

P1191**Board Number: B530****Chemical labelling of primary endocytic vesicles quantifies flux through clathrin coated pits, caveolae, and alternate endocytic pathways.**E. Shvets¹, V. Bitsikas¹, I. Correa², G. Howard¹, C. Hansen³, B. Nichols¹;¹MRC-LMB, Cambridge, England, ²New England Biolabs, Ipswich, MA, ³UCSD, La Jolla, CA

Several different endocytic pathways have been proposed to function in mammalian cells. Clathrin-coated pits are well characterised, but the identity, mechanism and function of alternative pathways have been less clear.

We applied chemical labelling of plasma membrane proteins to simultaneously define the origin and cargo composition of all primary endocytic vesicles. This was complemented by labelling of specific proteins with a reducible SNAP-tag substrate. These approaches provide high temporal resolution and stringent discrimination between surface-connected and intracellular membranes. Our data generate a complete and quantitative inventory of the different types of endocytic vesicle acting in unperturbed mammalian cells. Experiments employing mutated clathrin adaptors and loss of adaptor function allow analysis of the cargo load of endocytic vesicles when sorting of high affinity cargoes into clathrin coated pits is perturbed. These experiments reveal remarkably different effects on different classes of endocytic cargo.

Around 2% of endocytic vesicles arise from budding of caveolae from the plasma membrane, and these vesicles deliver cargo to endosomal compartments. Caveolae have a unique role in endocytosis, as both light and electron microscopy show that the caveolar bulb dynamically sorts plasma membrane components. The caveolar bulb acts as a molecular device that presents a different paradigm for sorting to the clathrin coated pit. These data provide new insight into the activity and diversity of endocytic pathways in mammalian cells.

P1192**Board Number: B531****A nanometer-scale survey of the structure and organization of clathrin-mediated endocytosis among mammalian cell lines.**K.A. Sochacki¹, J.W. Taraska¹;¹NHLBI/NIH, Bethesda, MD

Platinum replica electron micrographs (PREM) of unroofed mammalian cells provide an abundance of architectural detail about important cellular processes such as exocytosis, endocytosis, cytoskeletal organization, and adhesion. The topography of the cellular cortex and its spatial organization inform how the cell interacts and communicates with its surroundings. Structures like clathrin, non-clathrin vesicles, and actin are organized differently in different cell types reflecting their different functional

behavior. Here, we evaluate the size, shape, and spatial organization of these structures among 12 cell lines: PC-12 (rat adrenal gland), CHO-K1 (hamster ovary), HEK293 (human embryonic kidney), NIH/3T3 (mouse embryo), Cos7 (monkey kidney), HeLa (human cervix), INS-1 (rat pancreas), MCF7 (human breast), A549 (human lung), BS-C-1 (monkey kidney), U87 MG (human brain), and U2OS (human bone). High throughput image analysis using neural networks allows statistical analysis of cortex structures in several cells in each cell line. These data provide a systematic view of the plasma membrane molecular architecture in mammalian cells.

P1193

Board Number: B532

Internalization and Recycling of RET Receptor Tyrosine Kinase Isoforms.

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The RET (REarranged in Transfection) receptor tyrosine kinase is crucial for the normal development of the kidneys and enteric nervous system, as well as the regulation of neural-crest cell behaviours, including proliferation, migration, differentiation and survival. RET is also a potent contributor to clinically challenging cancers. Activating mutations in RET can lead to multiple endocrine neoplasia type 2, a cancer syndrome characterized by medullary thyroid carcinoma. Increased activation of wildtype RET receptors has been linked to aggressive breast cancers, and enhanced invasion and perineural spread in pancreatic carcinoma. Two distinct isoforms of the growth factor receptor arise through alternative splicing of the 3' exons, and are termed RET9 and RET51 to denote the number of amino acids in each C-terminal tail. These isoforms induce unique phosphorylation patterns on intracellular tyrosine residues, differentially bind downstream signalling proteins, and possess different intrinsic abilities to cause cellular transformation. Here, we explore the differential subcellular localization and trafficking of the two isoforms in human cell line models to better understand RET-mediated cancers.

We have found that RET51 is efficiently matured and localized to the cell membrane, while immature RET9 matures more slowly, leading to lower relative expression on the plasma membrane. Upon RET activation, both isoforms at the cell surface are internalized to endosomes via AP2-adaptor interactions and the clathrin coated-pit pathway. Although RET51 is internalized more rapidly than RET9, a portion of RET51 molecules recycle back to the plasma membrane, maintaining high protein levels at the cell surface. We have shown that RET51 interacts with the sorting protein GGA3, which may promote localization to recycling compartments. Recycling of RET51 leads to a longer residency time in an endosomal compartment, where sustained Erk1/2 can occur. Differences in signaling potential may in part explain the greater transforming potential of RET51. Together, our data suggest that differences in RET isoform subcellular localization and trafficking would lead to differences in signal transduction and may in part explain the functional differences observed between RET isoforms.

P1194**Board Number: B533****Identification of a Novel Complex Required for WASH-dependent Receptor Trafficking.**

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Intracellular sorting and trafficking pathways require a complex orchestration of events for proper distribution of internalized cargo proteins. Retromer is necessary for the successful sorting and trafficking of cellular cargoes to both the plasma membrane and the Golgi. An important regulator of receptor trafficking by the retromer is the Wiskott-Aldrich Syndrome Protein and SCAR Homolog (WASH) complex, an Arp2/3 Type 1 nucleation promoting factor which induces branched F-actin networks at the endosome surface (Gomez and Billadeau 2009). The WASH complex is composed of five subunits: SWIP, Strumpellin, FAM21, WASH1, and CCDC53, interacts with the retromer via FAM21, and plays a role in both retrograde receptor trafficking to the TGN as well as plasma membrane transport of receptors. Herein, we characterize a novel protein complex containing COMMD proteins, CCDC22, CCDC93 and C16orf62 (referred to as the CCC complex) that localizes to endosomes in a WASH complex-dependent manner. Significantly, depletion of any CCC complex component impairs recycling and intracellular trafficking of receptors regulated by the WASH and retromer complexes, including α -5 integrin, CI-M6PR, and the copper transporter ATP7A.

P1195**Board Number: B534****Role of plasma membrane-bound sialidase NEU3 in clathrin-mediated endocytosis.**M. Rodríguez-Walker¹, A.A. Vilcaes¹, J.L. Daniotti¹;¹Univ Nacional de Cordoba-CIQUIBIC (CONICET-UNC), Fac. Ciencias Quimicas, Cordoba, Argentina

Gangliosides (GS) are sialic acid-containing glycolipids expressed on plasma membranes from nearly all vertebrate cells. They have been implicated in many physiological and pathological processes, e.g. in cell growth, cell adhesion and endocytosis; including its capacity to function as receptors for several toxins, viruses and antibodies. GS are synthesized at the Golgi complex, but recently a number of enzymes of GS anabolism and catabolism have been shown to be associated with the plasma membrane. NEU3, a key plasma membrane-bound sialidase for GS hydrolysis, has been implicated in similar cellular processes as GS, and its up-regulation has been observed in various human cancer cells. In the case of clathrin-mediated endocytosis, although this has been widely studied, the specific role of GS and NEU3 in this cellular process has not yet been well established. Using biochemical assays and confocal microscopy imaging, we studied the role of GS and NEU3 in different endocytic processes. Initially, we observed an increase in the internalization of transferrin (Tf), the archetypical cargo for internalization through clathrin-mediated endocytosis, in cell lines expressing GS with high level of sialylation. The ectopic expression of NEU3 led to a drastic decrease in Tf endocytosis, suggesting a participation of GS in this process. However, expression of NEU3 in GS-depleted cells only slightly affected its inhibition on Tf endocytosis, indicating that NEU3 modifies the internalization of Tf independently of its action on GS. Additionally, the internalization of low-density lipoprotein, another typical ligand in clathrin-mediated endocytosis, was also decreased in NEU3-overexpressing cells. In contrast, internalization of cholera toxin β -subunit, which is endocytosed by both clathrin-dependent and clathrin-independent mechanisms, remained unaltered. Kinetic assays carried out on NEU3 expressing cells revealed a significant reduction in the sorting of Tf to early and recycling endosomes, without significant changes recorded in the expression level of the Tf receptor. We found that NEU3 expression altered the subcellular distribution of clathrin adaptor AP-2 σ_2 subunit, but did not reveal any changes in the membrane distribution of clathrin or phosphatidylinositol (4, 5)-biphosphate. Finally, NEU3 did not alter the distribution of caveolin-1. Overall, these results suggest a specific and novel role of NEU3 in clathrin-mediated endocytosis.

P1196**Board Number: B535****Palmitoylation is required to recruit retromer to endosomes.**G. Modica¹, F. Jules¹, A. Simoneau¹, H. Wurtele¹, S. Lefrançois¹;¹Hopital Maisonneuve-Rosemont, Montreal, QC

Palmitoylation is a reversible enzyme-mediated post-translational modification consisting of the covalent attachment of palmitic acid to a cysteine residue of a target protein. Within the last 10 years, a group of enzymes that contain a conserved catalytic DHHC domain have been shown to have palmitoyltransferase (PAT) activity. So far, 24 PATs have been identified in humans while the yeast *S. Cerevisiae* genome has 7. The deletion of two yeast PATs, Swf1 and Akr1, results in the abnormal extracellular secretion of the vacuolar protein Carboxypeptidase Y (CPY), suggesting a defect in the vacuolar sorting machinery. The correct sorting of CPY requires the efficient recycling of its vacuolar protein sorting receptor Vps10 from the endosome to the trans-Golgi network (TGN). This intracellular trafficking pathway is mediated by retromer, a pentameric protein complex consisting of a trimer composed of Vps26, Vps29 and Vps35 and a dimer made by Vps5 and Vps17. We found that Vps10 is degraded in Swf1-deleted cells as it cannot recycle to the TGN. Furthermore, we demonstrated that the deletion of Swf1 leads to a mislocalization of retromer subunits from the membrane fraction to a cytosolic fraction, supporting a role for this PAT in sorting at the endosome. Subsequently, using Acyl-RAC (Resin-Assisted Capture), we found that Vps29 is palmitoylated, suggesting an association between palmitoylation and retromer recruitment and function. Interestingly, Ypt7, the Rab protein mediating the recruitment of retromer to endosomal membranes, is also palmitoylated, but in a Swf1-independent manner. We found that the enzyme responsible for Ypt7 palmitoylation is the vacuole associated PAT Pfa3. While the deletion of Swf1 leads to the mislocalization of retromer and to the secretion of CPY, the deletion of Pfa3 does not. This may suggest that Ypt7 palmitoylation per se is not required for correct retromer function. Altogether our data indicates that palmitoylation plays a complex role in the recruitment of retromer to endosomes and is required for the efficient endosome-to-Golgi trafficking of Vps10.

P1197**Board Number: B536****Role of the clathrin independent, CLIC/GEEC (CG) endocytic pathway in membrane homeostasis.**J.J. Thottacherry¹, A. Kosmalska^{2,3}, A. Elosegui-Artola^{2,3}, R. Vishwakarma⁴, P. Roca-Cusachs^{2,3}, S. Mayor¹;¹National Centre for Biological Sciences (NCBS), TIFR, Bangalore, India, ²Institute for Bioengineering of Catalonia (IBEC), Barcelona, Spain, ³University of Barcelona, Barcelona, Spain, ⁴Indian Institute of Integrative Medicine, bangalore, India

Eukaryotic cells have multiple endocytic pathways that help internalize nutrients, ligands, receptors and extracellular fluid. Endocytic pathways and subsequent recycling are also important for rapid membrane

redistribution during processes such as cell spreading, mitosis and cell migration¹. Cells need to tightly coordinate membrane exchange through these endo-exocytic processes for the maintenance of its plasma membrane area and cell shape. Membrane tension has been thought to play a role in coordinating the exo-endocytic delivery of membrane to and from the cell surface. However, the nature of the endocytic pathway(s), molecules or mechanism(s) behind these regulated processes remains largely unknown.

Unlike the extensively studied clathrin mediated endocytic pathway, clathrin independent endocytic (CIE) pathways in particular the CLIC/GEEC (CG) pathway could serve as a central player in the homeostasis of membrane tension or area. The CG pathway is a high capacity CIE pathway that internalize the equivalent of entire PM area in 12 minutes². The CG pathway also rapidly regurgitates a major fraction of endocytosed contents within 5 minutes of internalization³. These properties give it the potential to be a core regulator of membrane dynamics that could also respond to membrane tension.

Here we have explored the role of multiple endocytic pathways in mammalian cells using fluorescent microscopy during de-adhering or upon changing tension using a custom built stretching device. We find that CG endocytic pathway is specifically up regulated during de-adhering while caveolar or clathrin mediated endocytic pathways are not involved. Changing membrane tension by stretching or via other means triggers similar response from CG pathway. Modulating the CG pathway via perturbation of key genes that regulate this pathway appear to modify the membrane tension, indicating the pathway could help set the resting membrane tension or maintain area homeostasis of a cell.

References:

Howes, M.T., Mayor, S. & Parton, R.G., 2010. Molecules, mechanisms, and cellular roles of clathrin-independent endocytosis. *Current opinion in cell biology*, 22(4), pp.519–527.

Howes, M.T. et al., 2010. Clathrin-independent carriers form a high capacity endocytic sorting system at the leading edge of migrating cells. *The Journal of cell biology*, 190(4), pp.675–91.

Chadda, R. et al., 2007. Cholesterol-sensitive Cdc42 activation regulates actin polymerization for endocytosis via the GEEC pathway. *Traffic*, 8(6), pp.702–17.

P1198

Board Number: B537

Defining the endocytic function of Pan1 in *Saccharomyces cerevisiae*.

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Endocytosis is a well-conserved process by which cells invaginate small portions of the plasma membrane to create vesicles containing extracellular and transmembrane cargo proteins. Dozens of proteins and hundreds of specific binding interactions coordinate and regulate these events.

Saccharomyces cerevisiae is a powerful model system to study clathrin-mediated endocytosis (CME). Pan1 is thought to act as a scaffolding protein due to its interactions with several proteins that act throughout the endocytic process. Previous research on Pan1 has characterized some of Pan1's binding interactions, but due to Pan1's essential nature, the exact mechanisms of Pan1's function in endocytosis have been difficult to define. To clarify Pan1's roles, I have developed a conditional PAN1 auxin-inducible degron allele (Pan1-AID) using the methods of Nishimura et al. (2009). In Pan1-AID, Pan1 is specifically and acutely degraded upon the switch to nonpermissive conditions. Cell growth and endocytosis are arrested within minutes after Pan1 depletion and the cells begin to die after one to two hours. We can learn more about Pan1's endocytic and essential functions by studying the phenotypes of the arrested cells immediately after growth arrest has been achieved and before cell death. Using Pan1-AID, I have more clearly defined Pan1's mechanistic role in CME as a critical regulator of the transition from the early endocytic coat to the late coat. Additionally, I determined a key role for Pan1's central region in endocytosis and viability. I have identified three overlapping essential regions of Pan1, all of which contain a portion of the central region. All three regions are able to localize to endocytic patches, but only one is able to partially restore endocytosis, suggesting Pan1 has an alternative essential role in the cell. Lastly, I will use Pan1-AID to identify Pan1's essential function.

P1199

Board Number: B538

A ROLE OF OCRL IN CLATHRIN-COATED PIT DYNAMICS AND UNCOATING REVEALED BY STUDIES OF LOWE SYNDROME CELLS.

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Loss of function mutations in OCRL, an inositol 5-phosphatase, are responsible for Lowe syndrome and type 2 Dent disease. OCRL has a widespread localization within cells, primarily in the endocytic pathway, including very late-stage clathrin-coated pits. However, the precise function of OCRL in the dynamics of endocytic clathrin coats remains unclear. Mass spectrometry proteomics of OCRL immunoprecipitates strongly emphasized a link of the OCRL interactome to clathrin-dependent membrane traffic. Endocytic clathrin coat components revealed by this approach included SNX9, which we have found to be a novel direct interactor of OCRL. SNX9, which also interacts with clathrin and other endocytic factors, is implicated in the late stages of endocytosis. In Lowe Syndrome fibroblasts, which completely lack OCRL, we observed an increase in the number of endocytic clathrin-coated pits and a significant delay in their turnover. We also found that many components of endocytic clathrin coats, including SNX9, clathrin and AP-2, accumulated on intracellular vesicles nucleating actin tails, which have been previously described

as a hallmark cellular phenotype of Lowe syndrome fibroblasts. Expression of GFP-OCRL in patient cells reversed these phenotypes, while a catalytically inactive GFP-OCRL accumulated at both endocytic clathrin-coated pits and at the head of actin tails, indicating the dependence of tails on abnormal and ectopic PI(4,5)P₂ accumulation. We established that at least a fraction of vesicles driving tail nucleation originated from the plasma membrane, suggesting that they are clathrin-coated vesicles that failed to shed the clathrin coat. We had previously shown that the PI(4,5)P₂ phosphatase synaptojanin, the major inositol 5-phosphatase at the synapse, plays a major role in the uncoating of clathrin coated vesicles at synapses. The present study suggests that OCRL may participate in clathrin uncoating during endocytosis in non-neuronal cells. Defects in clathrin-mediated endocytosis may contribute to the clinical manifestations of Lowe Syndrome.

P1200

Board Number: B539

PtdIns(4,5)P₂ homeostasis in endosomal trafficking is controlled by the Rab35 GTPase and the Lowe syndrome phosphatase OCRL.

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The membrane phospholipid phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P₂] is major regulator of actin dynamics and activates complexes involved in actin polymerization. Our laboratory has recently shown that the Rab35 GTPase recruits and directly interacts with the PtdIns(4,5)P₂ 5-phosphatase OCRL in late cytokinesis bridges during cytokinesis. This is essential for a normal abscission by preventing local PtdIns(4,5)P₂ and actin accumulation in the intercellular bridge connecting the daughter cells (1,2,3). Mutations in OCRL are responsible for the Oculo-Cerebro-Renal Syndrome of Lowe, a rare genetic disease characterized by renal proximal tubular dysfunction, which likely results from defects in the endosomal pathway (4). How OCRL is localized to endosomes remains a key question. Since the Rab35 GTPase is located on the endosomal system (1) we hypothesized that Rab35 may recruit OCRL on endosomes to prevent actin accumulation at their surface, by analogy with our results during cytokinesis. Remarkably, the levels of lysosomal enzymes in the plasma and urine are increased in Lowe patients, suggesting defects in the intracellular trafficking of the cation-independent mannose 6-phosphate receptor (CI-MPR) (4,5,6). Our study suggests that Rab35 critically controls the timing of OCRL recruitment on clathrin-coated endosomes, and is essential for PtdIns(4,5)P₂ hydrolysis and consequently normal trafficking in the endosomal system.

(1) Kouranti et al., *Current Biology* 2006 (2) Dambournet et al., *Nature Cell Biology* 2011 (3) Chesneau et al., *Current Biology* 2012 (4) Vicinanza et al., *EMBO J.* 2011 (5) Ungewickell et al., *PNAS* 1999 (6) Norden et al., *Nephrol Dial Transplant* 2008

P1201**Board Number: B540****Cross-talk between clathrin-mediated and clathrin-independent endosomal membrane systems coordinated by Rab35 and Arf6.**D. Dutta¹, J.G. Donaldson¹;¹Cell Biology and Physiology Center, NIH/NLHBI, Bethesda, MD

Clathrin-mediated endocytosis (CME) and clathrin-independent endocytosis (CIE) occur in most cells raising questions as to whether the two processes are coordinately regulated. In HeLa cells, we have identified an elaborate endosomal sorting system that sorts CIE cargo proteins on endosomes that also contain CME cargo proteins. This led us to investigate whether trafficking of CIE cargo proteins would be affected when CME is blocked. Using different approaches of inhibiting CME, we found that when CME is blocked, endocytosis by CIE continues but the intracellular trafficking of CIE cargo proteins is altered. CIE cargo proteins that normally traffic directly into Arf6-associated tubules after internalization and avoid degradation (CD44, CD98 and CD147), now traffic to lysosomes and are degraded. The endosomal tubules are also absent and the cargo proteins now visit endosomes associated with EEA1 on their way to the lysosomes. The altered trafficking and loss of the tubular endosomal network caused by inhibition of CME was rescued by expression of Rab35, a Rab associated with clathrin-coated vesicles, or by expression of the Rab35 effector ACAP2, an Arf6 GAP that inactivates Arf6. Indeed, Arf6-GTP levels were elevated in cells where CME was inhibited but were subsequently lowered in cells expressing Rab35. The other Rab protein associated with tubular endosome formation, Rab22, or other effectors of Rab35 and Arf6, MICAL-L1 failed to prevent the mis-sorting of CIE cargo proteins in the absence of CME. These observations indicate that Rab35 and Arf6 activities must be carefully regulated on endosomes and this regulation may be mediated by the mutual antagonism of Rab35 and Arf6 in that they each recruit GAPs that inactivate the other. With Rab35 marking CME input, loss of CME and thus Rab35 leads to elevated Arf6-GTP and shifts the sorting of CIE cargo proteins to lysosomes and degradation.

P1202**Board Number: B541****FCHo2 regulates protein assembly and efficiency of clathrin-mediated endocytosis.**E. Evergren¹, H. McMahon¹;¹Medical Research Council Laboratory of Molecular Biology, Cambridge, England

Clathrin-mediated endocytosis is important for receptor uptake, synaptic vesicle recycling, virus internalisation and tissue morphogenesis. Many of the proteins interactions involved in clathrin coated vesicle formation are mediated by short linear interaction motifs (SLiMs). The presence of multiple motifs within intrinsically disordered regions (IDRs) and the self-polymerization of clathrin in turn provide an ability to build large complexes necessary for vesicle formation. This implies a dynamic

process involving the assembly but also exchange of interacting partners with a functional efficiency, as assessed by uptake of ligands and receptors, based on the stoichiometry of components within the network. Whilst this is an established principle underpinning signal transduction it is so far unexplored in understanding clathrin coat formation. We hypothesize that endocytosis can be altered either by effects on recruitment or on the stability of recruited complexes. In this study we focus on FCHo2 and comprehensively define the motifs regulating its association with interacting proteins, discovering three novel motifs which mediate the binding of clathrin, AP2, Eps15R, Eps15, epsin1, CALM and intersectin-1. We then study the impact of disrupting these interactions on important subcomplexes en route to functional endocytic uptake of transferrin. Disruption has two important phenotypic outcomes: separation of protein subcomplexes from each other, or coalescing subcomplexes into larger complexes that do not mature. Adaptor proteins AP2 and FCHo2 bind to overlapping motifs in Eps15 via weak affinity interactions, and interactions between Eps15-Eps15R-FCHo2-AP2 function cooperatively to strengthen the complex in network modules. By changing their concentration a significant impact was found both in complex formation, colocalisation in cells, and the efficiency of ligand uptake. On the basis of our findings, we propose a model where colocalisation and protein complex formation of high-avidity low-affinity interactions between clathrin, FCHo2, AP2, Eps15 and Eps15R are fundamental to establishing efficient maturation of clathrin-coated pits. These cooperative interactions are sensitive to protein concentration. In cell-lines with different endogenous expression levels this was reflected in efficiency of transferrin uptake. Our study highlights the importance of assessing the impact of targeting a single endocytic adaptor protein on network organisation and protein complex formation rather than a single endpoint phenotype. It is well known in cell signalling that construction of higher order complexes is instrumental in building dynamic and yet robust processes. Here we show that similar principles also apply to the efficient assembly of clathrin coats.

P1203

Board Number: B542

Identifying Small Molecule Inhibitors of Epsin-Mediated Cargo Internalization.

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The Notch Signaling Pathway is involved in several essential functions, such as tissue development and renewal. Misregulation of the pathway leads to a variety of diseases, including developmental syndromes and Cancer. Epsin (Epn), an endocytic adaptor, mediates the internalization of notch ligands, (e.g., Delta: DLL1), which is the crucial step to trigger the activation of the notch signaling pathway. Therefore, epn-mediated cargo internalization is a suitable target for therapeutic development. The goal of this project is to identify compounds that interfere the internalization of notch ligand by epn. We have recently identified a motif responsible for Epn-specific recognition of the yeast Na-transporter ENa1. This motif is bound by Epn via conserved determinants, and homologous motifs were found in known human Epn cargoes such as DLL1. Importantly, the DLL1 motif was able to mediate the epn-dependent internalization of ENa1 when inserted in the transporter sequence. Therefore, we developed

a highly efficient and low cost FACs based compound screening system in *Saccharomyces cerevisiae*. Briefly, since the vesicles containing internalized ENa1-GFP fuse with the lysosome/vacuole and the cargo is degraded, decrease of cell-associated GFP fluorescence under promoter repression conditions reports the levels of cargo internalization. Using cell sorting we compared the proportion of cells with low-mid-high fluorescence intensity in samples exposed to vehicle and compounds from a library of pharmacologically active compound (LOPAC). This strategy led to identify 44 compounds with negligible toxicity and potential activity against ENa1-DLL1 internalization (interestingly, we also found 33 candidates capable of enhancing cargo internalization). We are currently elucidating the mechanism by which the candidate compounds inhibit Ena1 internalization. We anticipate that these results will allow us to establish the foundations for developing therapies against diseases caused by misregulation of the Notch signaling pathway.

P1204

Board Number: B543

Loss of adaptor protein-3 complex leads to defective Vangl2 trafficking and disruption of planar cell polarity and convergent extension.

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Planar cell polarity (PCP) regulates coordinated orientation of cells along an axis parallel to the plane of the epithelium. It is critical in development and tissue function. Defects in PCP result in a range of developmental anomalies and diseases including disorganization of auditory hair cells in the sensory epithelium of the cochlea, deafness, neural tube closure defects, and cancer. PCP proteins are asymmetrically sorted across the planar polarity axis in the mammalian inner ear which plays a crucial role to regulate coordinated hair cell polarity and cochlear duct extension. However, the molecular mechanisms that regulate the asymmetric localization of several essential PCP transmembrane proteins remain largely unknown. To elucidate the underlying mechanisms for Vangl2 asymmetric membrane partition and dissect its role in PCP signaling, a 2-hybrid screen was performed to identify novel Vangl2-interacting proteins. From this screen, an adaptor protein (AP) component, u2 was identified as an interacting protein with Vangl2. Co-immunoprecipitation and co-localization experiments were performed and confirmed a biochemical interaction between Vangl2 with AP-2, as well as with AP-3. These adaptor proteins complexes are known to be associated with vesicles along the endocytic route. The biochemical association of Vangl2 with AP-2 and AP-3 suggest that Vangl2 may be an AP cargo that traffics through the endosomal pathways. Live imaging revealed that Vangl2 is indeed associated with early and recycling endosomes as evident by co-localization with Rab5 and Rab21, as well as Rab11 and Rab4, respectively. Furthermore, Vangl2 membrane targeting is inhibited in cells treated with inhibitors that block early and recycling endosomal pathways. In AP-3 depleted cells, Vangl2 localization is disrupted and Vangl2 is no longer enriched at cell membrane. We examined the distribution of a core

PCP protein, Fz3 and discovered reduced membrane localization of Fz3 in mocha mice, which are AP-3 deficient. We observed the presence of additional rows of hair cells in the basal turn and length of the cochlea suggesting convergent extension deficits. Vestibular analysis of mocha animals revealed a disruption in polarity in hair cell orientation in the posterior cristae. Mocha mice are deaf and balance deficient which is consistent with the morphological abnormalities. These findings strongly suggest that AP-3 mediated endosomal pathways are essential for the membrane targeting of core PCP proteins. In vivo studies will be further employed to determine whether the asymmetric localization of Vangl2 is dependent upon AP-2 and/or AP-3 mediated endosomal pathways.

P1205

Board Number: B544

Acute regulation of blood-brain barrier glucose permeability.

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The human brain uses glucose as its major energy source, consuming up to 75% of total daily glucose intake. The blood-brain barrier (BBB), however, forms a permeability barrier that prevents simple diffusion of substrates from blood circulation into the brain. Thus, glucose must be transported across the BBB endothelial cells (ECs) by the glucose transporter, GLUT1. Misregulation of BBB GLUT1 expression or function results in severe cognitive and developmental disorders. Until recently, BBB ECs were viewed as a passive conduit for the uptake of glucose into the brain, with GLUT1 expressed predominantly at luminal and abluminal cell surfaces to facilitate trans-cellular glucose transport. Our lab has recently shown that AMP-activated protein kinase (AMPK) mediates the acute regulation of GLUT1-dependent sugar transport in brain microvascular ECs. Acute AMPK activation increases cell surface localization of GLUT1, and glucose transport, without increasing total GLUT1 mRNA or protein expression. This suggests that AMPK controls GLUT1 trafficking between intracellular and cell surface membranes. We want to understand how AMPK regulates GLUT1-mediated glucose transport in BBB ECs. To accomplish this, we are using a reversible cell surface biotinylation technique to study the kinetics of GLUT1 trafficking in the presence or absence of AMPK activation. This will reveal whether GLUT1 endocytosis or exocytosis is controlled by AMPK and will aid in the identification of downstream signaling partners mediating AMPK regulation of GLUT1 trafficking. Our preliminary data in basal, murine brain endothelial (bEnd.3) cells show that approximately 3% of plasma membrane GLUT1 and transferrin receptor (TfR) is internalized over 20 minutes at 37°C, whereas cell surface Na-K ATPase is not internalized. Conversely, we observe up to 70% net internalization of TfR in HEK-293 cells after a 20-minute incubation at 37°C. GLUT1 expression in HEK-293 cells is very low, so we plan to investigate GLUT1 trafficking using HEK-293 cells stably expressing human GLUT1. Future studies will address whether the low net internalization of GLUT1 and TfR in control bEnd.3 cells reflects low unidirectional internalization or rapid re-externalization of internalized GLUT1 and TfR. We will also determine how AMPK activation affects the rate constants for GLUT1 and TfR endocytosis and exocytosis, and the signaling pathway involved in this process. Findings from this study will provide fundamental insights

into cerebral glucose transporter misregulation in diseases and may reveal mechanisms for pharmacologic manipulation of the BBB permeability.

P1206

Board Number: B545

Coordinated action of ubiquitin ligases promote multivesicular body sorting of membrane protein receptors.

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The function of plasma membrane proteins may be tuned by altering their residence time on the plasma membrane and their transit time in the endocytic pathway prior to lysosomal degradation. Ubiquitin modification ensures recognition and association of membrane proteins with endosomal protein complexes that mediate the sorting of proteins to be degraded from those to be recycled. The precise nature of coordination between the cellular machineries that govern ubiquitination and endosomal sorting is not well understood. Lack of a ubiquitin tag precludes epidermal growth factor receptor (EGFR) from being sorted into MVB internal vesicles, and therefore from lysosomal targeting and degradation. While ubiquitination of EGFR may occur early in the endocytic pathway at or near the plasma membrane, it is not sufficient to promote MVB sorting that leads to receptor degradation. We observed that an E3 ubiquitin ligase, UBE4B, associated with endosomes by binding directly to the ESCRT-0 component Hrs. We analyzed the mechanism by which UBE4B regulates the multivesicular body sorting, and ultimately degradation, of EGFR. However, UBE4B is not the only E3 ligase that is associated with endosomes and the ESCRT complexes, nor is it the only E3 ligase that affects the degradation of EGFR. Here we show that coordinated, sequential action of endosomally-associated E3 ligases contribute to the sorting and degradation of plasma membrane proteins that travel through the endocytic pathway.

P1207

Board Number: B546

RNF167 ubiquitinates Arl8b to control cargo trafficking to lysosomes.

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Ubiquitination regulates a broad range of cellular processes, including endocytosis and endosome trafficking. A group of transmembrane RING finger proteins are localized to the endomembrane system

and are presumed to function as E3 ubiquitin ligases. To understand the function of these ubiquitin ligases, the identification of the substrates is of critical importance. Using proximity-directed biotin labeling technology, we devised an approach to identify substrates of a ubiquitin ligase. We applied this method to RNF167 and identified Arl8B as a potential substrate. We demonstrated that RNF167 ubiquitinates Arl8B at the lysine residue K141 and downregulates its expression. Overexpression of RNF167 caused delayed trafficking of EGF and dextran to lysosomes, indicating that RNF167 affected both receptor-mediated endocytosis and fluid phase pinocytosis. Coexpression of K141R mutant Arl8B abrogated inhibitory effects of RNF167 on cargo delivery to lysosomes, suggesting that RNF167 affected endosome trafficking by ubiquitination and subsequent degradation of Arl8B.

Endosomes, Lysosomes, and Lysosome–Related Organelles 2

P1208

Board Number: B547

Towards Understanding Cell-Scale Spatiotemporal Dynamics of Intracellular Transport.

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Intracellular transport is the process of distributing cargoes to specific destinations to meet the local metabolic and functional needs inside a cell. Given the dynamic and heterogeneous intracellular environment, a fundamental question regarding intracellular transport is how it is regulated to deliver the right cargo to the right place at the right time. So far, related studies have focused primarily on identifying and understanding the molecular machinery for transport of individual cargoes. However, the spatial and temporal control mechanism of intracellular transport, especially at the whole cell scale, remains poorly understood. To a great extent, this is due to the lack of methods for quantitative analysis of cell-scale spatiotemporal dynamics of intracellular transport. We propose an image based computational framework and related computational methods to characterize and analyze the cell-scale spatiotemporal dynamics of intracellular transport. We first developed an image processing technique to visualize the spatiotemporal dynamics of intracellular transport in an projected image. On top of that, machine learning techniques are used to identify patterns of the intracellular transport on the whole-cell scale. Specifically, we utilized the transport of fluorescently labeled lysosomes in BSC-1 cells as our model system. To mitigate the heterogeneity of cell shapes, micro-pattern contact printing technique were adapted to normalize cells into comparable conditions for systematic analysis. Our analysis revealed a few spatial patterns of lysosomal transport, suggesting the plausibility of global control mechanism on spatiotemporal distribution of lysosomes.

P1209**Board Number: B548****Mechanisms of membrane repair in polarized epithelia.**L. Tan^{1,2}, K.A. Toops^{1,3}, A. Lakkaraju^{1,2,3};¹Ophthalmology and Visual Sciences, University Wisconsin-Madison, Madison, WI, ²Pharmaceutical Sciences, University Wisconsin-Madison, Madison, WI, ³McPherson Eye Research Institute, Madison, WI

Maintenance of membrane integrity is critical for cell survival. Pore-forming toxins or the terminal complement complex C5b-9 create microlesions in membranes, leading to prolonged calcium influx and aberrant activation of signaling pathways that compromise cell function. Assembly of sub-lytic C5b-9 membrane attack complexes (MAC) on the plasma membrane of retinal pigment epithelial (RPE) cells contributes to the pathogenesis of age-related macular degeneration (AMD), the most common cause of irreversible blindness among older adults. Nucleated cells have multiple protective mechanisms to deal with sub-lytic MAC assembly including (i) expression of complement regulatory proteins such as CD59 to inhibit specific steps of MAC formation; (ii) elimination of assembled MAC by membrane shedding or by lysosomal degradation after endocytosis; and (iii) rapid resealing of membrane pores by the exocytosis of late endosomes and lysosomes. Here, we investigated which of these mechanisms operate in polarized monolayers of primary RPE using high-speed spinning disk and TIRF microscopy. Our data show that membrane integrity after sub-lytic MAC attack is maintained by the concerted actions of complement regulatory proteins and vesicle trafficking in the exocytic and endocytic routes. TIRF imaging using pHluorin-tagged constructs showed that synaptotagmin 7 participates in lysosome exocytosis after complement-mediated damage. We have previously demonstrated that lysosome exocytosis is predominantly basolateral in MDCK cells, due to the basolateral localization of syntaxin 4. In the RPE, syntaxin 4 is non-polar and as a result, lysosome exocytosis occurs both at the apical and basolateral domains. In RPE with lipofuscin bisretinoids or excess cholesterol, both of which are implicated in AMD pathogenesis, exocytic protective mechanisms are selectively impaired whereas endocytic mechanisms function efficiently. Cholesterol-mediated activation of acid sphingomyelinase in cells with bisretinoids induces microtubule acetylation, which interferes with the delivery of the GPI-anchored CD59 to the plasma membrane and inhibits lysosome exocytosis. Pharmacological inhibition of acid sphingomyelinase restores the protective mechanisms in cells with bisretinoids or excess cholesterol. Our studies provide insight into how membrane integrity can be compromised and restored in the face of multiple pathological insults in the RPE and suggest that precise organelle trafficking is critical for preventing chronic inflammation in the RPE and the retina.

P1210**Board Number: B549****Control of nutrient signaling and proteostasis by PI3K-C2-mediated PI(3,4)P2 synthesis.**A.L. Marat¹, W. Lo¹, V. Haucke¹;¹Leibniz Institut Fuer Molekulare Pharmakologie, Berlin, Germany

Eukaryotic cell function depends on a tight balance between biosynthetic growth promoting and degradative pathways. The mTOR pathway integrates signals elicited by growth and differentiation factors with the sensing of amino acids to promote protein synthesis and cell growth, while inhibiting lysosomal protein turnover and autophagy. Activation of mTOR signaling among other factors involves stimulation of class I phosphatidylinositol 3-kinase to synthesize phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P₃], which activates Akt to promote S6 kinase-mediated regulation of translation and shutdown of autophagy by phosphorylation of ULK1, as well as the nutrient stimulated generation of PI3P via the class III PI 3-kinase mVps34. How mTOR signaling is inactivated following nutrient and growth factor deprivation and whether and how this process is regulated by phosphoinositides is unknown. Here we identify class II phosphatidylinositol 3-kinase C2 (PI3K-C2) as a novel negative regulator of mTORC1 signaling. Depletion of PI3K-C2, an enzyme that specifically synthesizes phosphatidylinositol-3,4-bisphosphate [PI(3,4)P₂], promotes mTORC1 signaling and causes the dispersion and functional inactivation of LAMP1-positive late endosomes and lysosomes. This phenotype is rescued by co-depletion of the GTPase Arl8 or its effector SKIP, factors that crucially regulate lysosomal transport. Furthermore, there is a functional association of PI3K-C2 with the raptor subunit of mTORC1. Together, these data identify PI3K-C2-mediated PI(3,4)P₂ synthesis as a critical switch between growth promoting nutrient signals and lysosomal proteostasis, by negatively regulating mTORC1 signaling.

P1211**Board Number: B550****Ankyrin-B is required for directed cell migration and targeted recycling of β 1-integrin.**F. Qu¹, D.N. Lorenzo^{2,3}, V. Bennett^{4,5};¹Departments of Cell Biology and Biochemistry, Duke University, Durham, NC, ²Department of Biochemistry, Duke University Medical Center, Durham, NC, ³Howard Hughes Medical Institute, Durham, NC, ⁴Department of Biochemistry, Duke University, Durham, NC, ⁵Howard Hughes Medical Institute, Duke University, Durham, NC

Directed intracellular organelle transport is essential for many cellular processes, including cell migration. In migrating cells, the continuous recycling of β 1-integrin between plasma membrane and intracellular compartments facilitates cell adhesion and cell motility events. Ankyrin-B was recently

shown to promote microtubule-based multi-organelle transport by coupling dynactin to intracellular organelles through PIP3P. Here we report that ankyrin-B regulates the dynamic transport of β 1-integrin in mouse embryonic fibroblasts (MEF). We found that ankyrin-B null (AnkB^{-/-}) fibroblasts exhibited a ~50% delay in wound closure and deficits in directional cell migration on fibronectin substrates that were independent of alterations in the organization of the actin and microtubule-based cytoskeleton. Interestingly, loss of ankyrin-B resulted in disruption of β 1-integrin recycling to the plasma membrane and in accumulation of large intracellular β 1-integrin aggregates. To get a mechanistic insight into the role of ankyrin-B in promoting β 1-integrin recycling, we performed a series of structure-function studies by expressing ankyrin-B proteins lacking key residues or domains in ankyrin-B null MEFs. We show that the deficits in β 1-integrin recycling were fully rescued by expression of wild-type 220-kDa ankyrin-B or DAR975AAA ankyrin-B lacking β 2-spectrin-binding activity, and only partially restored by DD1320AA ankyrin-B lacking dynactin-binding activity. Surprisingly ankyrin-B proteins lacking the death domain completely lost their ability to localize to β 1-integrin positive compartments and failed to rescue β 1-integrin recycling. The death domain is highly conserved among members of the ankyrin family in different species but its function remains unknown. We demonstrate that ankyrin-B regulates the targeted trafficking of β 1-integrin and potentially other cargoes through a dynactin-dependent step working in concert with the heretofore uncharacterized death domain.

P1212

Board Number: B551

Polymerization of Clathrin by Ent5 Regulates the Timing of Maturation of Clathrin Coated Vesicles.

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Membrane traffic is an essential cellular process that delivers proteins and lipids to the right cellular compartments. Many protein cargos are delivered by clathrin coated vesicles (CCVs). The recruitment of clathrin to membranes is regulated by clathrin adaptors, which bind to lipids and cargo. Recruitment of clathrin by adaptors is thought to be sufficient for clathrin assembly. At the TGN and endosomes, two adaptors Gga2 and Ent5 bind directly to clathrin and also one another. These adaptors undergo stereotypical recruitment dynamics, where Gga2 always precedes Ent5. It is unclear why, if clathrin assembly only requires clathrin recruitment, Gga2 structures never form and mature in the absence of Ent5. Here we report that Ent5 clathrin binding is important for the maturation of Gga2 containing clathrin coats, thus preventing coats from maturing without Ent5. We find that Gga2 binds cooperatively to Ent5 and to clathrin. Clathrin binding stabilizes the interaction between Ent5 and Gga2. However, the interaction of Ent5 with clathrin but not with Gga2 is important to stabilize Ent5 at membranes. This finding suggests that direct interaction between Gga2 and Ent5 is not important for Ent5 recruitment. Similarly, Ent5 fails to co-purify with clathrin vesicles when Ent5 cannot bind to clathrin, suggesting that the interaction with clathrin is required for Ent5 to be incorporated into vesicles. Using time-lapse

microscopy, we demonstrate that when Ent5 is unable to bind to clathrin, the lifespans of Gga2 containing structures are prolonged and the recruitment of Ent5 is delayed significantly. This mutation causes a defect in the traffic of Chs3 at TGN and endosomes, indicating the delay is functionally important. Our findings suggest that the interaction between Ent5 and clathrin is the key for clathrin to polymerize into coated structures. We propose that the interaction between Ent5 and clathrin is an important check point for vesicle formation at TGN and endosomes. This interaction ensures that CCVs form in timely manner.

P1213

Board Number: B552

Macropinosome-lysosome fusion is a clathrin dependent process in bone marrow derived macrophages.

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Macrophages nonspecificly take up extracellular fluids, solutes and macromolecules by macropinocytosis. Understanding the mechanisms of macropinosome maturation will inform the study of lipid uptake, viral entry, antigen processing and presentation, as well as regulation of cell growth. Recently, we demonstrated that colony stimulating factor-1 receptor (CSF-1R) is internalized by small vesicle endocytosis, trafficked to nascent macropinosomes and degraded. These CSF-1R positive macropinosomes mature through a sequence similar to endosomes, progressing from EEA1 and Rab5 to Rab 7 positive vesicles before fusing with lysosomes. Here we report the assembly of large clathrin rings on internalized macropinosomes shown both by live-cell microscopy of clathrin light chain-yellow fluorescent protein (CLTA-YFP) overexpression and by immunostaining of endogenous clathrin heavy chain (CHC). Partial depletion of the clathrin heavy chain by siRNA prevented macropinosome-lysosome fusion and impaired degradation of the CSF-1R, with only minimal effects on the delivery of the CSF-1R to the macropinosome. Immunofluorescence staining of endogenous proteins demonstrates that clathrin assembled on macropinosomes co-localizes with the CSF-1R as well as ESCRT-0 member HRS, and homotypic fusion and protein sorting (HOPS) complex member VPS 39. These data indicate a novel role for clathrin in mediating macropinosome maturation and macropinosome lysosome fusion in macrophages.

P1214**Board Number: B553****Transport of the Macrophage Colony Stimulating Factor 1-Receptor into the Lumen of Macropinosomes.**L. Huang¹, N.W. Thiex², A.D. Hoppe¹, J. Lou¹;¹Chemistry and Biochemistry, South Dakota State Univ, Brookings, SD, ²Biology and Microbiology, South Dakota State Univ, Brookings, SD

The colony stimulating factor-1 receptor (CSF-1R) is a single pass type 1 membrane receptor. Upon ligand binding, it is internalized via small vesicles that are subsequently transported to macropinosomes in macrophages and is rapidly degraded. Using immunostaining against the both the N-epitope and C-epitope of CSF-1R, we demonstrate that upon arrival at the macropinosomes, CSF-1R is transported to the lumen of macropinosomes via a processes consistent with intraluminal budding. Hrs, a key member of the Endosomal Sorting Complex Required for Transport (ESCRT), co-localized with the CSF-1R on macropinosomes during this time. Furthermore, the ubiquitin ligases Cbl and Cbl-b were required for efficient transport of the CSF-1R to the macropinosomes and rapid co-localization with Hrs. Surprisingly, Cbl and Cbl-b are not required for CSF-1R degradation. In primary bone marrow macrophages derived from Cbl/Cbl-b double knock out animals, CSF-1R degradation takes place in a non-macropinosome derived compartment. In conclusion, targeting of the CSF-1R to macropinosomes via ubiquitination by Cbl and Cbl-b followed by Hrs assembly on macropinosomes leads to the rapid degradation of activated CSF-1R and mediates an important deactivation step in CSF-1R signal transduction.

P1215**Board Number: B554****The Legionella pneumophila effector protein LegC7 alters yeast endosomal trafficking.**K. O'Brien¹;¹Microbiology, University of Georgia, Athens, GA

Legionella pneumophila are obligate intracellular pathogens that invade environmental amoebae as well as human alveolar macrophages resulting in the severe form of pneumonia known as Legionnaires' disease. In order to avoid lysosomal degradation, Legionella secretes a large number of effector proteins via a Type IVb secretion system into the host; some of these proteins manipulate a myriad of host membrane trafficking events to create the membrane-bound replicative niche, termed the Legionella-containing vacuole (LCV). Using the model system, Saccharomyces cerevisiae, we seek to define the effects and targets of one such effector protein, LegC7, previously shown to be toxic when expressed in yeast and causing an apparent Class E vacuolar protein sorting phenotype. In this work, we identify a point mutant in the second coiled coil domain of LegC7 that results in greatly decreased toxicity, indicating that the second coiled coil domain of LegC7 is crucial for either target binding or

proper protein folding. We also show that LegC7 disrupts vacuolar delivery of both biosynthetic and endocytic cargo yet does not disrupt endosome-independent pathways to the yeast vacuole. Furthermore, we demonstrate that a deletion of the ESCRT-0 complex member VPS27 causes a partial reversal of LegC7 toxicity; no other mutations of ESCRT pathway components display a similar phenotype. Taken together, we hypothesize that LegC7 functions to directly manipulate endosomal maturation in host cells, which could be exploited by the bacterium to help prevent phagosome-lysosome fusion during infection.

P1216

Board Number: B555

Inducible expression of MCOLN2 in immune cells.

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Mucolipins (or TRPMLs) constitute a family of endosomal cation channels with homology to the transient receptor potential superfamily. In mammals, the mucolipin family includes three members, mucolipin-1, -2, and -3 (MCOLN1-3). MCOLN1 and -3 have been well characterized. However, the cellular function of MCOLN2 remains elusive. Consistent with the proposed role of MCOLNs in Ca²⁺-dependent membrane trafficking, we have previously described that recombinant MCOLN2 enhances recycling of internalized GPI proteins back to the cell surface in HeLa cells. To further corroborate MCOLN2 function in a more physiologically relevant cell type, we analyzed MCOLN2 expression in a series of mouse tissues and organs by RT-PCR. We found that MCOLN2 was expressed predominantly in lymphoid and kidney organs. Notably, expression of MCOLN2 was tightly regulated at the transcriptional level. While MCOLN2 was present at low levels in resting RAW 264.7 macrophages, its expression was induced over 10 fold in response to Toll-like Receptors (TLRs) activation. In contrast, the levels of MCOLN1 and MCOLN3 did not change upon TLR activation. To confirm the upregulation of MCOLN2 in response to TLR activation in primary cells we isolated bone marrow macrophages, alveolar macrophages, and microglia from mice and treated them with a panel of TLR activators, including Zymosan (TLR2 ligand), PolyIC (TLR3 ligand), LPS (TLR4 ligand), R-848 (TLR7/8 ligand), and Imiquimod (TLR7 ligand). In all cases, we observed a significant increase in the levels of MCOLN2 upon TLR activation as assessed by RT-PCR, western-blot, and immunofluorescence. Endogenous MCOLN2 co-localized to perinuclear vesicles that also contain transferrin receptor and likely correspond to recycling endosomes. This is in clear contrast with MCOLN1 and MCOLN3 that mainly localize to the late and early endosomal pathway, respectively. Overall, our data reveal interesting differences in the regulation and distribution of the members of the MCOLN family and suggest a possible role of MCOLN2 in innate immune response.

P1217**Board Number: B556****Distinct Intracellular Trafficking Patterns of Host IgG by Herpes Virus Fc-Receptors.**B. Ndjamen¹, S.E. Fraser², P.J. Bjorkman^{1,3};¹Biology and Biological Engineering, California Institute of Technology, Pasadena, CA, ²Translational Imaging Center, University of Southern California, Los Angeles, CA, ³Investigator, Howard Hughes Medical Institute, Pasadena, CA

Members of both alpha and beta herpes viruses affects 50–98% of people around the world. They cause severe symptoms in congenitally infected newborns, a lifelong latent infection that is lethal in immunocompromised individuals, and are associated with several types of cancer. Human cytomegalovirus (HCMV) and herpes simplex virus type 1 (HSV-1) viruses express proteins (HCMV gp68 and gp34; HSV-1 gE-gI) that function as Fc receptors (FcRs) by binding to the Fc regions of human IgG. In addition to binding free IgG, these viral FcRs can bind to IgG complexed with an antigen to form an antibody bipolar bridged (ABB) complex. Although HCMV gp68 and HSV-1 gE-gI have an overlapping binding site on Fc, the finding that the gp68/Fc interaction is stable at pH values between 5.6 and 8.1 but that gE-gI binds only at neutral or basic pH suggests distinct pH-based downstream events after IgG is internalized via receptor-mediated endocytosis into intracellular compartments. Here we developed a cell-based in vitro model system to define the fates of ABB complexes formed by the two types of viral FcRs. We found that alpha (HSV-1) and beta (HCMV) herpes virus FcRs displayed distinct intracellular trafficking patterns to target internalized ligands: HSV-1 gE-gI dissociates from its IgG-antigen ligand in acidic endosomal compartments and recycles back to the cell surface, whereas HCMV FcRs (gp68) are transported together with IgG-antigen complexes to lysosomes for degradation. In both cases, anti-viral IgGs and their viral targets are selectively degraded, a potential immune evasion strategy allowing herpes viruses to escape from IgG-mediated immune responses.

P1218**Board Number: B557****Differential regulation of organelle dynamics by tubulin acetylation in polarized epithelia.**K.A. Toops^{1,2}, L. Tan^{1,3}, A. Lakkaraju^{1,2,3};¹Ophthalmology and Visual Sciences, University Wisconsin-Madison, Madison, WI, ²McPherson Eye Research Institute, Madison, WI, ³Pharmaceutical Sciences, University Wisconsin-Madison, Madison, WI

Intracellular trafficking is coordinated by the actin and microtubule cytoskeletons and associated motor proteins. Organelle-specific recruitment of motor proteins is accomplished in part by post-translational modifications of α -tubulin such as acetylation and detyrosination. Here, we used high-speed live imaging to monitor organelle dynamics in the prototypical polarized MDCK cell line and in adult primary retinal

pigment epithelial (RPE) monolayers. A key function of the RPE is the daily phagocytosis and degradation of shed photoreceptor outer segments, necessary for photoreceptor health and for vision. Over a lifetime, this high metabolic activity leads to the accumulation of visual cycle by-products called lipofuscin bisretinoids within the RPE endo-lysosomal system. Lipofuscin bisretinoids have been implicated in the pathogenesis of numerous retinal diseases including age-related macular degeneration, the most common cause of vision loss among older adults today. We have shown that bisretinoids trap cholesterol and bis(monoacylglycero)phosphate, an acid sphingomyelinase cofactor in RPE lysosomes. Acid sphingomyelinase activation increases cellular ceramide, which promotes tubulin acetylation on stabilized microtubules. Live imaging using spinning disc confocal microscopy revealed that long-range displacement of LC3-labeled autophagosomes and LAMP2-labeled late endosomes/lysosomes (LE/Lys) is significantly impaired in cells with abnormally acetylated microtubules. This results in incomplete autophagic flux and accumulation of canonical autophagic substrates. Calcium-induced fusion of LE/Lys with the plasma membrane is critical for membrane repair and removal of cellular debris. In RPE with lipofuscin bisretinoids or in MDCK with excess cholesterol, we observed decreased LE/Lys exocytosis after exposure to calcium ionophores or pore-forming toxins. Therefore, constrained LE/Lys trafficking due to tubulin acetylation interferes with both autophagy and membrane repair. Inhibition of acid sphingomyelinase decreased ceramide and acetylated tubulin levels, restored autophagy and LE/Lys exocytosis in RPE and MDCK cells. In contrast to its effect on LE/Lys and autophagosome motility, tubulin acetylation accelerated the trafficking of rab11-labeled apical recycling endosomes. This was associated with increased apical secretion of exosomes containing flotillin-1. Our data show that tubulin acetylation decreases transport of organelles in the degradative route and increases trafficking of organelles in the recycling route. Studies are currently underway to elucidate how selective recruitment of motors and scaffolding proteins drives organelle-specific motility in polarized epithelia and how this might contribute to disease processes.

P1219

Board Number: B558

Regulation of E-Cadherin Recycling by Discs Large (hDlg).

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E-cadherin is single pass type –I transmembrane glycoprotein that is localized to the adherens junction and basolateral membrane in epithelial cells and consists of a large extracellular domain, a transmembrane segment, and a conserved cytoplasmic domain. b-catenin is at the cytoplasmic site of the cell and binds to the intracellular domain of E-cadherin and is also complexed with a-catenin bound to actin. This adhesive pool ultimately links the cytoskeletal networks of adjacent cells, contributing to the maintenance of normal tissue architecture. The adhesive function of E-cadherin plays a vital role in epithelial physiology and changes in the level of cell-cell adhesion molecules are crucial for the development of aggressive carcinomas.

Control of epithelial architecture is a complex process involving also the coordinate activity of three multi-molecular signaling complexes: the Crumbs complex, the Par complex and the Scrib complex. The human hScrib complex consists of three proteins, hScrib, hDlg1 and Hugl-1, and loss of either Scrib or Dlg produces imaginal discs overgrowth and an invasive phenotype in *Drosophila*. In human cells, hScrib and hDlg1 appear to regulate important pathways governing cell polarity and cell attachment. Recently these proteins have been demonstrated to be involved in different signaling pathways and more interestingly also in vesicle trafficking inside the cell.

Since previous studies have demonstrated that perturbation of the scribe/hDlg module can impact negatively upon E-cadherin localization, we were interested in investigating how the hScrib/hDlg module affects E-cadherin trafficking. Using a variety of approaches we show that loss of hDlg results in a substantial decrease in membrane bound E-cadherin. Furthermore, this does not appear to be due by changes in the steady state levels of E-cadherin, but rather is a reflection of alteration in E-cadherin recycling. Current studies are focused on defining molecular mechanisms by which hDlg can modulate endosomal transport.

P1220

Board Number: B559

The cyclin-dependent kinase, Pho85/Cdk5 regulates the synthesis of the signaling lipid phosphatidylinositol 3,5 bis phosphate.

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Cyclin dependent kinases (CDKs) are key regulators of the cell cycle, cellular nutrient homeostasis, cell polarity and gene expression. Here we show an unexpected link between a CDK and an endosomal signaling lipid, phosphatidylinositol 3,5 bis phosphate (PtdIns(3,5)P₂). In *Saccharomyces cerevisiae*, PtdIns(3,5)P₂ is generated on endocytic organelles by the PtdIns(3)P 5-kinase, Fab1 and functions as a signaling molecule in multiple pathways including regulation of TORC1. PtdIns(3,5)P₂ is found in all eukaryotes and defects in its synthesis underlie multiple neurological diseases. Physiological signals cause rapid, and transient changes in PtdIns(3,5)P₂ levels. However, it was not known how the levels of PtdIns(3,5)P₂ are regulated during acute environmental change. Here we report results from a genetic screen for mutants defective in vacuole inheritance (*vac* mutant). Characterization of a subset of these mutants, as well as others, led to the discovery that the CDK, Pho85 and the cyclin, Pho80 are required for the acute elevation of PtdIns(3,5)P₂ upon hyper-osmotic shock. Pho85-Pho80 directly phosphorylate Fab1 and positively regulate the synthesis of PtdIns(3,5)P₂. Furthermore, we find that the phosphorylation of Fab1 may lead to a conformation change that activates its lipid kinase activity. Cdk5 and p35, mammalian homologue of Pho85 and Pho80, respectively, are particularly critical in neuronal physiology. Notably, we find that mammalian Fab1 (PIKfyve) is a direct target of Cdk5-p35 in tissue culture cells. Our studies reveal an upstream pathway that regulates the synthesis of PtdIns(3,5)P₂ through CDK and further show that this mechanism is conserved in yeast and mammalian cells.

P1221**Board Number: B560****Parkinson's Disease Causing Mutations Alters Retromer's Function.**J. Follett¹, S. Norwood¹, N. Hamilton¹, B. Collins¹, A. Bugarcic¹, R.D. Teasdale¹;¹Institute for Molecular Bioscience, University of Queensland, Brisbane, Australia

Mammalian Retromer is a protein complex composed of three proteins Vps26, Vps29 and Vps35, with an important role in the sorting and trafficking of transmembrane receptors within the endosome. The retromer complex forms a stable trimer, which associates with a range of other proteins, including sorting nexins (SNXs), which modulate its function. Recently, a pathogenic point mutation within the Vps35 subunit (D620N) was linked to the manifestation of Parkinson's disease (PD). Our initial characterization of the PD-linked Vps35 D620N mutant defined the impact this mutation has on Retromer's function in both model cell lines and fibroblasts isolated from a PD patient. Our recent work has focused on the contribution of retromer to cellular properties associated with PD including the formation of α -synuclein aggregates in neuronal cell lines which is increased when retromer levels are reduced in the cell. In addition, we have examined if other PD associated mutations within the three-retromer proteins have a detrimental effect on its function. For example, we find that expression of Vps35 R524W mutant disrupts the trafficking of cathepsin D, a CI-M6PR ligand and that this subunit is not able to efficiently interact with multiple sorting nexins.

P1222**Board Number: B561****Trafficking of moonlighting Glyceraldehyde-3-phosphate dehydrogenase to membrane and extracellular milieu is mediated via multiple non-classical secretory pathways.**A. Chauhan¹, H. Malhotra¹, N. Sheokand², M. Kumar¹, P. Jakhar¹, C. Raje³, M. Raje¹;¹Cell Biology and Immunology, Institute of Microbial Technology, Chandigarh, India, ²Cell Biology and Immunology, Inst Microbial Technology, Chandigarh, India, ³Biotechnology, National Institute of Pharmaceutical Education and Research, Mohali, India

Numerous pathological conditions like iron starvation, infection and cancer have been shown to be associated with the enhanced trafficking of the cytosolic moonlighting protein glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to plasma membrane (PM) and also its secretion into extracellular milieu (ECM). One of its moonlighting functions at PM and in ECM involves the trafficking of the iron carrier proteins transferrin and lactoferrin into mammalian cells. However the exact molecular mechanism behind the recruitment of this cytosolic protein to PM and ECM is not known. In the current study we report that trafficking of GAPDH involves multiple pathways of non-classical protein secretion. Our results demonstrate that trafficking of GAPDH does not involve classical ER-Golgi pathway of protein

secretion as its presentation to the PM and ECM is not affected by brefeldin A. This finding is in accordance to the fact that GAPDH lacks any secretory signal. Further we report that enzymatically active GAPDH is secreted by cells in exosome free and exosome bound forms. Exosomes are nano vesicular structures enclosed in a larger assembly known as multivesicular bodies (MVBs). Presence of GAPDH inside the MVBs and exosomes was confirmed by co-localization confocal microscopy, western blotting and GAPDH specific enzyme activity. Modulation of the exosomal secretory pathway using pharmacological agents was shown to modulate GAPDH expression on PM and its release into ECM. We confirmed that GAPDH transport via this pathway accounts for 35% of the total secreted GAPDH. As an alternative pathway, we report that GAPDH trafficking also involves the secretory lysosomal pathway. We confirmed the presence of GAPDH in secretory lysosomes by confocal microscopy. Finally we confirmed the involvement of lysosomal secretory pathway in GAPDH trafficking to PM and ECM utilizing various small molecules like ATP, NH_4Cl , and MgCl_2 which affect lysosomal exocytosis. Translocation of GAPDH into these secretory lysosomes was found to be dependent on ABC transporters present in lysosomal membrane. Using live cell imaging and fluorescence recovery after photobleaching (FRAP) experiments we find that newly synthesized GAPDH is selectively recruited to the vesicles and these vesicles are transported towards the plasma membrane. Collectively our findings suggests for the first time that GAPDH is trafficked to PM and ECM via lysosomal and exosomal non-classical secretory pathways.

P1223

Board Number: B562

Lysosomal delivery modulates the toxicity of aminoglycoside antibiotics in mechanosensory hair cells.

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The zebrafish lateral line system is a useful model for the study of mechanosensory hair cells. In the inner ear cochlea, hair cells detect mechanical stimulation caused by sound waves and convert physical movement into nerve impulses. These impulses are then conducted through the auditory system. Hair cell damage underlies the majority of hearing loss. While zebrafish do have an ear organ, they also have another sensory system that uses hair cells to detect external movements in their environment—the lateral line (LL). Many compounds known to cause hearing loss also kill lateral line hair cells. Unlike inner ear hair cells, LL hair cells are superficial and are therefore accessible and easy to observe. We have studied the response of LL hair cells to aminoglycosides—a family of antibiotics including neomycin and gentamicin that are well known to damage hearing. Here we look at how aminoglycosides enter LL hair cells, and identify trafficking events that modulate their hair cell toxicity. Aminoglycosides (AGs) can be fluorescently tagged without significantly altering their behavior. Fluorescent analogs of AGs reveal that initial AG loading occurs in the apical region of hair cells. This entry is gated by mechanotransduction (MET). Entry is blocked in both mutants that lack MET activity, and in wild type hair cells treated with known MET blocking agents like amiloride. AG entry can be perturbed by a diverse array of agents

including selective estrogen receptor modulators, a beta-adrenergic agonist, and quinoline ring derivatives. Most but not all AG uptake-blocking agents appear to act by altering MET activity. Following entry, aminoglycosides distribute into two visually distinct pools—a diffuse population present throughout the cell body, and bright membrane-bound punctae. Notably, AGs that rapidly kill hair cells distribute primarily to the diffuse pool. Those that kill hair cells more slowly distribute primarily to punctae. These punctae colocalize with lysosomal markers including Rab7 and LysoTrackers. Interfering with dynamin-dependent endocytic processes blocks the initial formation of punctae and increases the diffuse signal. However, slower lysosomal delivery still occurs through a pathway sensitive to 3-methyladenine treatment. Delaying lysosomal delivery of AGs increases aminoglycoside toxicity. Our trafficking studies indicate intracellular pools that differentially contribute to AG toxicity, and suggest that rapid damage from AGs likely comes from the diffuse pool, not the punctae. Delivery to the punctae may initially be cytoprotective by sequestering aminoglycosides. AG delivery to lysosomes can be monitored, and accelerating this delivery may provide a means to reduce the hair cell toxicity of AGs.

P1224

Board Number: B563

Spastic paraplegia proteins spastizin and spatacsin mediate autophagic lysosome reformation.

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Autophagy allows cells to adapt to changes in their environment by coordinating the degradation and recycling of cellular components and organelles to maintain homeostasis. Lysosomes are critical organelles for terminating autophagy via their fusion with mature autophagosomes, generating autolysosomes that degrade autophagic materials. Therefore, maintenance of the lysosomal population is essential for autophagy-dependent cellular clearance. We demonstrate that the two most common autosomal recessive hereditary spastic paraplegia gene products, the SPG15 protein spastizin and SPG11 protein spatacsin, play pivotal roles in autophagic lysosome reformation (ALR), a pathway that generates new lysosomes. Lysosomal targeting of spastizin requires its intact FYVE domain, which binds phosphatidylinositol 3-phosphate. Moreover, loss of spastizin or spatacsin results in depletion of free lysosomes, which are competent to fuse with autophagosomes, as well as an accumulation of autolysosomes, reflecting a failure in ALR. Mechanistically, spastizin and spatacsin are essential components for the initiation of lysosomal tubulation. These findings uncover a specific link of the autophagy/lysosomal biogenesis machinery to neurodegeneration.

P1225**Board Number: B564****Retromer stabilization partially and selectively rescues sphingolipid dysregulation in Niemann-Pick C disease models.**B. Zhou¹, C. Dall'Armi¹, R.B. Chan¹, Y. Yang¹, C. Marquer¹, D.E. Berman¹, S.L. Sturley², G.A. Petsko³, A.B. Munkacsy⁴, S.A. Small⁵, G. Di Paolo¹;¹Department of Pathology Cell Biology, Columbia University Medical Center, New York, NY, ²Institute of Human Nutrition, Columbia University Medical Center, New York, NY, ³Department of Biomedical Engineering, Weill Cornell Medical College, New York, NY, ⁴Victoria University of Wellington, Wellington, New Zealand, ⁵Department of Neurology, Columbia University Medical Center, New York, NY

Niemann-Pick C disease (NPC) is a neurovisceral lipid storage disorder with a broad clinical spectrum typically comprising systemic dysfunction followed by variable onset of fatal, progressive neurodegeneration. NPC is transmitted in an autosomal recessive fashion by mutations in either *NPC1* or *NPC2* genes, which encode proteins thought to play a role in lysosomal export of free cholesterol. However, precise pathophysiological mechanisms are unclear, and no disease-modifying treatment exists. Here, we first provide a comprehensive lipidomic characterization of over 30 lipid subclasses including sterols, glycerolipids, glycerophospholipids, sphingolipids, and sphingoid bases in *Npc1*^{-/-} mice using state-of-the-art HPLC-coupled mass spectrometric analysis, unmasking heretofore undetected brain and liver lipid changes, most notably in dihydrosphingolipid metabolism. We further confirm these changes in U18666A drug-induced NPC1 models and in NPC1 patient-derived fibroblasts. Finally, we reverse a subset of sphingolipid changes through drug-induced stabilization of the retromer complex and then complement these findings with biochemical and confocal microscopy analyses. Hence, we provide new insight regarding the lipid pathways perturbed in NPC and offer evidence for a novel therapeutic strategy.

P1226**Board Number: B565****Characterization of human KCTD9: A novel autophagosome-associated protein.**F. Ladha¹, X. Teng¹, J. Hardwick¹;¹W. Harry Feinstone Department of Molecular Microbiology and Immunology, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD

The human KCTD9 belongs to the K⁺ channel tetramerization domain-containing (KCTD) protein family and is an uncharacterized protein commonly deleted in cancers and proposed to mediate natural killer (NK) cell activation in chronic hepatitis B virus (HBV) infections. Our lab found the yeast homologue of the KCTD9, Whi2, is required to inhibit TOR kinase, and also required to activate autophagy. The KCTD/Whi2 proteins are very poorly studied, but contain a BTB domain and therefore have been proposed to act as adaptors for the E3 ligase Cullin-3, similar to other BTB-containing proteins. We sought to determine KCTD9's subcellular localization and its involvement in the autophagy pathway.

For the first time, we were able to confirm that KCTD9 forms cytoplasmic puncta, which localize to autophagosomes. KCTD9 puncta did not co-localize with endosomal markers, but specifically co-localized with autophagy proteins LC3, Vps34, and Atg5. In addition, KCTD9 associates to endoplasmic reticulum (ER) markers. These results suggest KCTD9 is a novel autophagosome-associated protein that potentially localizes to subdomains of the ER from which autophagosomes derive from. Further investigations will allow us to determine if KCTD9 plays a functional role in the autophagy pathway.

P1227

Board Number: B566

A unique PDZ domain and arrestin-like fold interaction reveals mechanistic details of endocytic recycling by SNX27-retromer.

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The sorting nexin-27 (SNX27)-retromer is a major regulator of endosome-to-plasma membrane recycling of trans-membrane cargoes that contain a PDZ binding motif. Here we describe the core interaction in SNX27-retromer assembly and its functional relevance for cargo sorting. Crystal structures and nuclear magnetic resonance experiments reveal that an exposed β -hairpin in the SNX27 PDZ domain engages a groove in the arrestin-like structure of the VPS26A retromer subunit. The structure establishes how the SNX27 PDZ domain simultaneously binds PDZ binding motifs and retromer-associated VPS26. Importantly, VPS26A binding increases the affinity of the SNX27 PDZ domain for PDZ binding motifs by an order of magnitude, revealing co-operativity in cargo selection. With disruption of SNX27 and retromer function linked to synaptic dysfunction and neurodegenerative disease, our work provides the first step in the molecular description of this important sorting complex, and more broadly describes a unique interaction between a PDZ domain and an arrestin-like fold.

P1228

Board Number: B567

Conformational changes in Ist1 lead to distinct modes of Vps4 regulation.

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The Endosomal Sorting Complexes Required for Transport (ESCRTs) mediate membrane remodeling processes that bud vesicles away from the cytoplasm or that dissect membrane tubules from within the structures. ESCRT-III in particular has been implicated in this membrane remodeling. Disassembly of ESCRT-III by the AAA-ATPase Vps4 is required for ESCRT function, however it remains unclear how Vps4 activity is regulated to permit proper ESCRT-III function. Ist1 is unique among the ESCRT-III subunits in

that it is able of both inhibiting and stimulating Vps4 ATPase activity, suggesting Ist1 may be an important regulator of Vps4. We have characterized three distinct modes of Vps4 regulation by Ist1 in vitro: (1) hyper-stimulation requiring the Ist1 MIT-interacting motif 1 (MIM1) element, (2) a lower level of MIM1-independent stimulation, and (3) inhibition requiring both the MIM1 element and a highly conserved ELYC motif. Furthermore, using analytical gel filtration and limited proteolysis, we demonstrate that conformational changes in Ist1 impacts modes of Vps4 regulation. Finally, in vivo analyses in yeast support a functional role for the putative Ist1 inhibitory and stimulatory domains. These observations suggest that Ist1 regulation of Vps4 changes as Ist1 is recruited to the ESCRT-III polymer and conformational changes occur.

Rab GTPases

P1229

Board Number: B568

Tracking Incipient Functional Diversification in the Rab GTPase Gene Family.

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As membrane trafficking determinants, Rab GTPases drive fusion between subcellular compartments in a highly specific manner. As a result, during eukaryotic evolution, Rab duplication and diversification have paralleled the elaboration of trafficking pathways. While the most conserved Rabs appear to have conserved functions across all eukaryotic species, others are quite plastic, diversifying rapidly in different lineages. In an effort to understand both the biochemical and evolutionary sources of Rab plasticity, we are combining the wealth of existing structure-function data from previous Rab biochemical studies with sequence information and localization data from a large closely-related species complex of unicellular ciliates, the *Paramecium aurelias*. First, we have identified a subset of ciliate Rabs that are highly structurally conserved, particularly in key effector binding domains, when compared by homology modeling to crystal structures of orthologous human, mouse or yeast Rabs. These structurally conserved ciliate Rabs also share identical localization patterns with their orthologs, despite being only ~50% identical in amino acid sequence. Second, we have identified a set of ciliate Rabs that appear to be undergoing incipient diversification, i.e. they fall within conserved subfamilies phylogenetically, and have retained the conserved Rab backbone structure, but appear to be undergoing large conformational changes in their effector binding regions. We are in the process of localizing these putatively diversifying Rabs; preliminary work suggests that they now localize to new or different trafficking pathways when compared to more conserved Rabs in the same subfamily. By examining the nature of these modeled binding domains, and the amino acid changes that have accompanied them on a large scale (across 15 species and in several different subfamilies), we aim to better understand the 'rules' for Rab plasticity, and how they are able to bind new effectors and occlude others in the process of switching to new trafficking pathways in the cell.

P1230**Board Number: B569****The ER-resident Rab8A GTPase is involved in the trafficking of surface proteins necessary for phagocytosis in the enteric protozoan parasite *Entamoeba histolytica*.**Y. Hanadate^{1,2}, K. Nakada-Tsukui¹, T. Nozaki^{1,2}, Y. Saito-Nakano¹;¹National Inst Infectious Diseases, Tokyo, Japan, ²University of Tsukuba, Ibaraki, Japan

The phagocytosis is initiated by the specific recognition and attachment of ligands via plasma membrane receptors. During maturation, series of intracellular compartments, such as the ER, endosomes, and lysosomes, are associated with phagosomes. Engulfed particles are then subsequently degraded by the specific hydrolases. In these events, Rab GTPases play pivotal roles in membrane fusion and regulated transport. *Entamoeba histolytica*, an intestinal protozoan parasite, engulfs bacteria and host cells. Thus, phagocytosis is indispensable for the pathogenesis of *E. histolytica*. Interestingly, *E. histolytica* codes more than 100 Rab genes in their genome, whereas *Homo sapiens* possesses around 60 Rab genes despite of their multicellularity. Biological functions of most of Rab GTPases are still poorly understood in *E. histolytica*.

In our previous phagosomal proteome analysis, we identified 14 Rab proteins from the amoebic phagosomes. Among them, a homologue of Rab8, which plays a role in trafficking from the trans-Golgi network to the plasma membrane in other organisms, was isolated. This work is the first report that Rab8 participates in phagocytosis. First, small antisense RNA-mediated transcriptomic gene silencing (gs) was used to examine the physiological role of EhRab8A in phagocytosis. Down-regulation of EhRab8A caused remarkable reduction in phagocytic efficiency of erythrocytes, bacteria, and carboxylated latex beads. This was attributable to the reduced efficiency of adhesion to targets. Surface-biotinylation showed that the presentation of several surface proteins that may be involved in recognition of targets was reduced in EhRab8A-gs strain compared to the wild-type. Upstream of Rab8 lies the guanine nucleotide exchange factor (GEF), Rabin8, which is also identified as Rab11 effector in *Homo sapiens*. Thus, the colocalization of Rab11 and Rab8 in the recycling endosomes was often reported in other organisms. However, in *Entamoeba*, EhRab8A was not colocalized with EhRab11B, a representative isotype of four Rab11 proteins. Instead, EhRab8A was localized to the proximate region of the ER in the steady state. Accordingly, EhRab8A might have role in independent pathway from EhRab11B. These results indicate that EhRab8A regulates the receptor sorting at proximate region of the ER to the plasma membrane.

P1231**Board Number: B570****Rab32 is Essential for Maintaining Functional Acidocalcisomes and for Growth and Virulence of *Trypanosoma cruzi*.**S. Niyogi¹, R. Docampo², V. Jimenez³;¹Cellular Biology, University of Georgia/CTEGD, Athens, GA, ²Center for Tropical and Emerging Global Diseases and Department of Cellular Biology, University of Georgia, Athens, GA, ³California State University, Fullerton, CA

Trypanosoma cruzi, the causative agent of Chagas disease, is a unicellular parasite that possesses a contractile vacuole complex (CVC) which consists of a bladder and a loose network of interconnected tubules known as the spongiome. Similar to its role in free-living protists where the CVC is usually present, this organelle in *T. cruzi* also plays a vital role in the regulation of the cell volume and in the response to osmotic stresses in all the life stages of the parasite. In addition, we recently reported a role for the CVC in trafficking of glycosyl phosphatidylinositol (GPI)-anchored proteins to the plasma membrane. When performing the proteomic analysis of the CVC of *T. cruzi* we detected the presence of Rab32 (TcRab32) in addition to several other proteins involved in vesicle trafficking and fusion activities. Mammalian Rab32 is involved in the biogenesis of lysosome-related organelles (LRO) and as LROs of human platelets and mast cells, acidocalcisomes (ACCs) have rounded morphology, are acidic, and rich in calcium, pyrophosphate (PPi) and polyphosphate (polyP). Therefore we investigated whether TcRab32 is involved in the transport of membrane proteins to the ACC. In this study we report that similar to most Rab-GTPases, TcRab32 is geranylgeranylated and also confirm its localization to the CVC. A dominant negative mutant tagged with GFP (GFP-TcRab32DN) localizes to the cytosol, and epimastigotes expressing this mutant have a defect in growth and in their ability to respond to osmotic stress conditions. Mutant parasites are still able to differentiate into metacyclic forms and infect host cells but they are less virulent than wild type cells. Parasites expressing GFP-TcRab32DN have ACCs of altered morphology, which are deficient in pyrophosphate and polyphosphate. Acidocalcisomes are less electron-dense in mutant epimastigotes, fewer in number and have a defect in proton transport. Taking together these results reveal that TcRab32 is important for acidocalcisome function, suggesting its involvement in trafficking of membrane proteins to these organelles. As TcRab32 is a contractile vacuole resident protein, this study provides the long searched link between the contractile vacuole and the ACC further demonstrating the role of the CVC as a trafficking hub.

P1232**Board Number: B571****HCV Inhibits Lysosomal Fusion Through Suppression of a Rab7 Interacting Protein.**A.L. Wozniak¹, K.N. Jones-Jamtgaard², R. Ralston¹, S.A. Weinman¹;¹Internal Medicine, University of Kansas Medical Center, Kansas City, KS, ²Microbiology, Molecular Genetics, and Immunology, University of Kansas Medical Center, Kansas City, KS

Hepatitis C virus (HCV) modulates intracellular trafficking pathways to serve viral replication and particle production and to evade cellular antiviral responses. The mechanisms of how this occurs and its consequences are poorly understood. The aim of this study was to determine if HCV-induced defects in autophagy are due to changes in Rab GTPase function. We have previously shown that during HCV infection, flux through the autophagy pathway is greatly diminished compared to control cells. To determine if HCV suppressed autophagosome-lysosome fusion events, control and HCV-infected cells were transfected with tandem RFP-GFP-LC3, where the quenching of the GFP fluorescence indicates autophagolysosome formation. In HCV infected cells, there was no loss of GFP fluorescence from puncta indicating lack of fusion between autophagosomes and lysosomes. We next performed fusion assays with isolated subcellular organelles to assess whether lack of fusion was intrinsic to autophagosomes and lysosomes themselves. Vesicles isolated from HCV-infected cells fused with each other normally in this in vitro fusion assay suggesting that the cellular fusion defect resulted from trafficking rather than inability of vesicles to fuse. To test if there were effects on other trafficking steps, we assessed EGF-induced receptor degradation. HCV inhibited this endosome-lysosome fusion as well. Since Rab7 is involved in both fusion processes, we assessed the effect of HCV on Rab7-dependent processes. HCV infection of Huh7.5 cells caused a dramatic increase in Rab7 immunofluorescence without changing Rab7 protein or GTPase activity levels by western blot. This suggested conformation or binding partner changes in Rab7. HCV infection reduced the level of the Rab7 effector protein, Rab-interacting lysosomal protein (RILP) compared to control. Knockdown of RILP to a similar level produced by HCV suppressed EGFR degradation in a nearly identical fashion as HCV infection. In conclusion, HCV infection suppresses Rab7-dependent trafficking events due to a decrease in the Rab7 effector, RILP. Loss of RILP-dependent Rab7 function may be a mechanism by which HCV promotes its lifecycle or subverts antiviral mechanisms. Future studies will focus on defining alterations in the Rab7 complex and trafficking due to loss of RILP.

P1233**Board Number: B572****Starvation-induced phosphorylation of the exchange factor DENND3 by Unc-51-like kinases activates Rab12 inducing macroautophagy.**J. Xu¹, P.S. McPherson¹;¹McGill University, Montreal, QC

Unc-51-like kinases (ULKs) are the most upstream kinases in the initiation of autophagy, yet the molecular mechanisms underlying ULK function in autophagy are essentially unknown. By investigating two poorly characterized proteins, the guanine nucleotide exchange factor (GEF) DENN domain-containing protein 3 (DENND3) and its substrate, the small GTPase Rab12, we have now discovered a novel mechanism accounting for ULK function in autophagy. We demonstrate that both DENND3 and Rab12 play positive roles in starvation-induced autophagy. Furthermore, under starvation, ULK1/2 phosphorylates DENND3 at two sites, activating Rab12 through upregulation of DENND3 GEF activity. By binding to LC3 and associating with autophagosomes, active Rab12 facilitates autophagosome trafficking. Together, our data reveal a novel pathway from starvation-induced ULK signaling through DENND3 to Rab12-mediated membrane trafficking required for autophagy.

P1234**Board Number: B573****A Novel Rab10 Regulated Complex Essential for the Autophagic Engulfment of Lipid Droplets.**Z. Li¹, R. Schulze², H. Cao², M.A. McNiven²;¹Mayo Graduate School, Rochester, MN, ²Mayo Clinic, Rochester, MN

Autophagic digestion of lipid droplets (LDs) is a central mechanism by which cells catabolize lipids as an energy source. Currently, the mechanisms by which autophagosomes (APs) physically bind and envelope LDs for subsequent digestion are poorly defined. In this study we report that the small GTPase, Rab10, implicated previously in the Golgi to plasma membrane transport of nascent secretory cargo, plays an essential role in the autophagy of LDs in hepatocytes. We find that Rab10 activity is amplified significantly in cells stimulated to undergo autophagy, concomitant with an increased association of this GTPase to the LD-AP interface or "synapse". Importantly, disruption of Rab10 function by siRNA knock down or expression of a GTPase defective protein leads to LD accumulation. Finally, activation of Rab10 during an autophagic stimulus increases its association with EHD2 and EHBP1, two effector proteins known to support membrane remodeling within the endocytic pathway. These proteins are recruited to the LD-AP synapse in a Rab10- dependent manner and are essential for autophagic-based LD breakdown. These findings identify a novel protein complex essential for the engulfment of LDs during the autophagic process.

P1235**Board Number: B574****Loss of Rab6 results in mis-sorting of Cathepsin D, impaired autolysosome function and defective nutrient sensing in the larval fat body of *Drosophila melanogaster*.**C.I. Ayala¹, T. Neufeld^{1,2};¹Genetics, Cell and Developmental Biology, University of Minnesota, Minneapolis, MN, ²Genetics Cell Biology and Development, University of Minnesota, Minneapolis, MN

Autophagy is a lysosome-dependent degradative pathway used by organisms to maintain quality control of organelles, proteins and energy homeostasis in cells. The pathway is negatively regulated by the mechanistic target of rapamycin (mTOR) under basal conditions, and requires elements of the Rab-dependent secretory pathway for autophagosome biogenesis and function. However, the interactions between autophagy, mTOR and Rab proteins remain incompletely understood *in vivo*. Here, we show that loss of Rab6 causes a development-dependent accumulation of autophagosomes, reduction in cell size and expansion of the lysosomal compartment. Characterization of these phenotypes revealed that they result primarily from mis-sorting of Cathepsin D from lysosomes, rendering them with a reduced degradative capacity upon autophagosome-lysosome fusion. This autolysosomal dysfunction leads to a defective recovery of mTOR activity, likely due to a reduced nutrient flux out of autolysosomes. Interestingly, these defects can be rescued by inactivation of the mTOR inhibitor PTEN, but not by re-feeding of exogenous nutrients nor constitutive activation of amino acid signaling, indicating an additional novel role for Rab6 in regulation of the mTOR signaling axis. Our findings suggest that loss of Rab6 interferes with the reciprocal regulation between autophagy and mTOR during distinct nutrient conditions by affecting two distinct traffic routes.

P1236**Board Number: B575****Influenza A Virus Assembly Intermediates Fuse in the Cytoplasm.**S. Lakdawala¹, Y. Wu², P. Wawrzusin², A. Broadbent¹, J. Kabat³, E. Lamirande¹, E. Fodor⁴, N. Altan-Bonnet⁵, H. Shroff², K. Subbarao¹;¹Laboratory of Infectious Diseases, NIH/NIAID, Bethesda, MD, ²Section on High Resolution Optical Imaging, NIH/NIBIB, Bethesda, MD, ³Research Technologies Branch, NIH/NIAID, Bethesda, MD, ⁴Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom, ⁵NIH/NHLBI, Bethesda, MD

Reassortment of influenza viral RNA (vRNA) segments in co-infected cells can lead to the emergence of viruses with pandemic potential. Replication of influenza vRNA occurs in the nucleus of infected cells, while progeny virions bud from the plasma membrane. Rab11a-containing recycling endosomes have

been implicated in the transport of vRNA from the nucleus to the plasma membrane. However, the intracellular dynamics of vRNA assembly into progeny virions is not well understood. Here we used recent advances in microscopy to explore vRNA assembly and transport during a productive infection. We visualized four distinct vRNA segments within a single cell using fluorescent in situ hybridization (FISH) and observed that foci containing more than one vRNA segment were found at the external nuclear periphery, suggesting that vRNA segments are not exported to the cytoplasm individually. Although many cytoplasmic foci contain multiple vRNA segments, not all vRNA species are present in every focus, indicating that assembly of all eight vRNA segments does not occur prior to export from the nucleus. To extend the observations made in fixed cells, we used a virus that encodes GFP fused to the viral polymerase acidic (PA) protein (WSN PA-GFP) to explore the dynamics of vRNA assembly in live cells during a productive infection. Since WSN PA-GFP colocalizes with viral nucleoprotein and influenza vRNA segments, we used it as a surrogate for visualizing vRNA transport in 3D and at high speed by inverted selective-plane illumination microscopy. We observed cytoplasmic PA-GFP foci colocalizing and traveling together en-route to the plasma membrane. Our data strongly support a model in which vRNA segments are exported from the nucleus as complexes that assemble en-route to the plasma membrane through dynamic colocalization events in the cytoplasm.

P1237

Board Number: B576

ER-coordinated activities of Rab22a and Rab5a drive uptake, endosomal compaction and intracellular processing of *Borrelia burgdorferi* by primary human macrophages.

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Borrelia burgdorferi is the causative agent of Lyme disease, a multisystemic disorder affecting primarily skin, joints and nervous system. Macrophages and dendritic cells counteract *Borrelia* dissemination in the human body through capturing, internalization and subsequent degradation of spirochetes. Here, we show that *B. burgdorferi* internalization by primary human macrophages involves compaction of spiral-shaped borreliae into endosomal vesicles. Compaction of borreliae is an active process that is driven by Rab22a and Rab5a, as shown by use of respective mutant constructs and siRNA-mediated depletion. RabGTPase-dependent trafficking is also important for maturation of borreliae-containing endosomes into LAMP1-positive and degradative lysosomes. Live cell imaging further shows that uptake of *Borrelia* is a multistep process, where Rab22a-positive endosomes enwrap elongated spirochetes, while endosomal compaction is mediated by contact with Rab5-positive vesicles at sites of membrane extrusion from endosomes. These sites are apparently coordinated by contact with the endoplasmic reticulum, as visualized by the ER marker Sec61beta. Collectively, these data identify Rab22a and Rab5a as crucial regulators of *B. burgdorferi* intracellular processing by primary macrophages. We also demonstrate that RabGTPase-driven endosomal compaction of borreliae is a crucial step in the vesicular

cascade that ultimately leads to elimination of spirochetes. Moreover, Rab22a and Rab5a exert their influence through discrete vesicular entities, whose activities are coordinated at ER contact sites.

P1238

Board Number: B577

Dysfunction of Rab3D is associated with increased secretion of tear cathepsin S, a tear biomarker of Sjögren's syndrome.

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Purpose. Rab3 and Rab27 are two subfamilies of Rab proteins identified in many cell types, including lacrimal gland acinar cells (LGAC), which facilitate secretory vesicle (SV) exocytosis. LGAC produce and release tear proteins to the ocular surface. We explored the role that changes in Rab3D and/or Rab27 may play in the altered tear cathepsin S (CTSS) levels observed in non-obese diabetic (NOD) mouse, a Sjögren's syndrome (SS) disease model. SS is a chronic autoimmune disease characterized by lymphocytic infiltration and damage to lacrimal and salivary gland, leading to severe dry eye and xerostomia. Increased CTSS abundance and activity in NOD LGAC and in tears were reported in an earlier study, as well as clinical studies validating CTSS as a tear biomarker of SS.

Methods. Mice deficient in Rab27a (*ashen*), Rab27b (27bKO), Rab27a and b (DKO), Rab3D (3DKO) as well as parent C57BL/6 (C57) were used, in addition to NOD, and BALB/c mice. qRT-PCR was performed with mRNA extracted from whole mouse lacrimal gland (LG) or LGAC obtained with laser capture microdissection. Tear fluid was collected from mice by stimulating LG with carbachol *in situ*, and the activities of several tear proteins were analyzed including CTSS and β -hexosaminidase (β -hex).

Results. Compared with C57, Rab27-deficient mice (*ashen*, 27bKO and DKO) had significantly lower CTSS and higher β -hex activities in tears. However, 3DKO mice had significantly higher CTSS but equivalent β -hex activities in tears. In NOD mice LGAC, Rab3D but not Rab27 gene levels were significantly lower than that in BALB/c. Gene expression of Rab3D effector dynein light chain was decreased, whereas Rab27 effectors MyosinVa and Munc 13-4 were increased. In NOD mouse, CTSS had both a higher gene expression level in LGAC and increased activity in mouse tears, whereas β -hex remained unchanged.

Conclusions. Some secretion of CTSS and β -hex occurs through both Rab27- and Rab3D-enriched SV. However, our data suggest that CTSS is secreted mainly through Rab27-enriched SV, while β -hex is secreted mainly through Rab3D-enriched SV. When exocytosis of one set of SV is impaired, proteins may be secreted from the other subset through compensatory mechanisms. In our mouse model, decreased expression of Rab3D but not Rab27 in LGAC causes the expression of CTSS, a SS tear biomarker, to increase. Studies from other groups have shown that Rab3D protein levels and protein distribution are altered in acinar cells from lacrimal and salivary glands from patients with SS. These findings collectively suggest that Rab3D dysregulation is implicated in increased secretion of the CtsS biomarker in disease and that both represent potential therapeutic targets.

P1239**Board Number: B578****Requirements of Rab5-activity, growth factor-dependent in highly invasive breast cancer cell lines.**N. Porther¹, M.A. Barbieri¹;¹Biological Sciences, Florida International University, Miami, FL

Rab5 expression in cancer has been associated with diseases progression and prognosis. We have previously shown that growth factor-directed cell invasion and migration was dependent on Rab5 activation in non- invasive breast cancer cells. However, hardly any data is available regarding the role of Rab5 in invasive cells in the absence or presence of growth factor. In our present study, we report that the invasive and migratory properties of the highly invasive breast cancer cell line, MDAMB-231, were abrogated in cells expressing the inactive (GDP-bound) form of Rab5 irrespective of growth factor stimulation; while the invasive potential of breast cancer cell lines expressing the wild type and active (GTP-bound) form of Rab5 were noticeably greater. Interestingly, expression of Rin1, a Rab5 GEF, increases invasion and migration of MDAMB-231 cells even in the absence of growth factors. In contrast, Rin1 depletion partially mitigates both processes. Hence, it is reasonable to speculate that cell invasion and migration, a process that typically involves activation of growth factor receptor, can be intracellularly regulated without intervention of activation of growth factor receptor

P1240**Board Number: B579****Phospho-activation of Rab8 Guanine Nucleotide Exchange Factor Rabin8 by ERK1/2 in Response to EGF Signaling.**J. Wang¹, J. Ren¹, B. Wu¹, S. Feng¹, W. Guo¹;¹University of Pennsylvania, Philadelphia, PA

Regulation of exocytosis is essential for many basic physiological processes ranging from hormone release to epithelia formation. Rab8, a member of the Rab family of small GTPases, plays an important role in membrane trafficking from the trans-Golgi network and recycling endosomes to the plasma membrane. Rabin8 is a guanine nucleotide exchange factor (GEF) and primary activator of Rab8. Investigating how Rabin8 itself is activated in cells is thus pivotal to the understanding of the regulation of exocytosis. Here we show that phosphorylation is an important mechanism for Rabin8 activation. We identified Rabin8 as a direct phospho-substrate of Extracellular Signal-Regulated Kinases 1 and 2 (ERK1/2) in response to epidermal growth factor (EGF) signaling. At the molecular level, ERK phosphorylation relieves the auto-inhibition of Rabin8, thus promoting its GEF activity towards Rab8. We further demonstrate that blocking ERK1/2-mediated phosphorylation of Rabin8 inhibits vesicular

trafficking to the plasma membrane. Together, our results suggest that ERK1/2 phosphorylation of Rabin8 plays an important role in exocytosis in response to extracellular signaling.

P1241

Board Number: B580

The small GTPase RAB-10 regulates autophagy function in *C. elegans*.

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Autophagy and endocytosis are two cellular pathways vital for development and tissue homeostasis. Autophagy is a catabolic process involving the formation of a double-membrane vesicle called the autophagosome that engulfs and degrades long-lived proteins and damaged organelles through the lysosomal pathway. Endocytosis involves the uptake and transport of cargo into the cell through the formation of single membrane vesicles termed endosomes. While several studies have shown crosstalk between autophagy and endocytosis, it is not clearly understood how specific endocytic compartments contribute to autophagy function. An example of crosstalk is the formation of an amphisome, the fusion of an autophagosome and an endosome, however the molecular mechanisms that regulate this fusion are not well understood. We have investigated the relationship between autophagy and endocytosis in *C. elegans*. We had previously shown that long-lived *daf-2* insulin-like/IGF-1 receptor (IIR) loss of function mutants have increased autophagy levels as measured by the formation of GFP::LGG-1 positive foci, a reporter for autophagosomes. If *daf-2/IIR* mutants are treated with RNAi against autophagy genes, the GFP::LGG-1 reporter aberrantly forms large aggregates. Thus, we screened for endocytic genes that, when depleted by RNAi, had a similar phenotype as the depletion of autophagy gene activity in *daf-2/IIR* loss of function mutants, that is the aberrant formation of GFP::LGG-1 large aggregates. We identified the small GTPase RAB-10, a mediator of basolateral endocytic trafficking in worms and mammals, as important for autophagy, particularly amphisome formation. We find that reduced activity of RAB-10, by RNAi or loss of function mutation, results in the formation of GFP::LGG-1 positive aggregates in *daf-2/IIR* mutants. Additionally, we find that RAB-10 depletion disrupts the colocalization of the GFP::LGG-1 positive foci and tagRFP::LMP-1 labeled lysosomes, suggesting that the autophagosomes in *rab-10* mutants are defective. Interestingly, under conditions that induce autophagy, such as *daf-2/IIR* loss of function, we observed an increase in the colocalization of GFP::RAB-10 positive endosomes and the autophagy marker mCherry::LGG-1, suggesting that RAB-10 endosomes fuse with GFP::LGG-1 positive autophagosomes. In summary, our findings suggest that RAB-10 is an important factor that coordinates endocytosis and autophagy during amphisome formation in *C. elegans*.

P1242**Board Number: B581****TLR sorting by Rab11 endosomes maintains intestinal epithelial-microbial homeostasis.**S. YU¹, Y. Nie^{2,3}, B. Knowles⁴, R. Sakamori¹, E. Stypulkowski⁵, C. Patel⁶, S. Das¹, V. Douard⁶, R.P. Ferraris⁶, E.M. Bonder¹, J.R. Goldenring⁷, Y.T. Ip^{2,3}, N. Gao^{1,8};¹Rutgers University, Newark, NJ, ²Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA, ³Department of Biological Sciences, Rutgers University, Newark, NJ,⁴Experimental Surgery, Vanderbilt University Medical Center, Nashville, TN, ⁵Cancer Biology, University of Pennsylvania, Philadelphia, PA, ⁶Department of Pharmacology and Physiology, Rutgers-New Jersey Medical School, Newark, NJ, ⁷Vanderbilt University School of Medicine, Nashville, TN, ⁸Rutgers Cancer Institute of New Jersey, New Brunswick, NJ

Compartmentalization of Toll-like receptors (TLRs) in intestinal epithelial cells (IECs) regulates distinct immune responses to microbes; however, the specific cellular machinery that controls this mechanism has not been fully identified. Here we provide genetic evidences that the recycling endosomal compartment in enterocytes maintains a homeostatic TLR9 intracellular distribution, supporting mucosal tolerance to normal microbiota. Genetic ablation of a recycling endosome resident small GTPase, Rab11a, a gene adjacent to a Crohn's disease risk locus, in mouse IECs and in *Drosophila* midgut caused epithelial cell-intrinsic cytokine production, inflammatory bowel phenotype, and early mortality. Unlike wild-type controls, germ-free Rab11a-deficient mouse intestines failed to tolerate the intraluminal stimulation of microbial agonists. Thus, Rab11a endosome controls intestinal host-microbial homeostasis at least partially via sorting TLRs.

P1243**Board Number: B582****Dual rab17 species have distinct biochemical properties.**A. Striz¹, P. Tuma¹;¹Department of Biology, Catholic Univ America, Washington, DC

Hepatocytes have a complex polarized membrane architecture which requires complex membrane trafficking pathways. These trafficking pathways are regulated, in part, by rab GTPases which are acylated for integration into lipid membranes and activated by GTP binding and hydrolysis. Particularly we have chosen to study rab17, whose expression is restricted to polarized epithelial cells and enriched in liver suggesting a role in the regulation of polarized protein trafficking. To initiate our studies, we generated three recombinant adenoviruses expressing wild type, constitutively active (GTP bound) or dominant negative (GDP bound) rab17. Like endogenous rab17, and as observed in other epithelial cell types, overexpressed wild type and the mutant rab17s were present at or near the apical membrane. Western blotting revealed immunoreactive species at 25 kDa (the predicted rab17 molecular weight) and 40 kDa. Mass spectrometry confirmed that both bands are rab17. When we expressed a

prenylation deficient rab17 isoform, the 40 kDa band was lost suggesting the shift in molecular weight is due, in part, to acylation. Because many rabs participate in vesicle docking with members of the SNARE machinery, and because rab17 has been shown to bind syntaxin 3 in kidney, we used GST pulldown assays with WIF-B cell lysates to analyze rab17-syntaxin interactions. We limited our studies to syntaxins 2 and 3 (the apical isoforms) and for our negative control, syntaxin 4 (the basolateral isoform). As predicted, syntaxin 4 did not bind wild type or the mutant rab17s. However, unlike in kidney, wild type and GTP bound rab17 bound syntaxin 2, not syntaxin 3. Interestingly, in both cases, only the 40 kDa rab17 species bound syntaxin 2 suggesting acylation is required for syntaxin binding indicating that the two forms have distinct binding properties. Blotting of total membrane fractions from WIF-B cells revealed that the 25kDa species is present in both the soluble and membrane fraction; however, the 40kDa species was detected only in the membrane fraction. We are currently investigating high molecular weight post-translational modifications (e.g., ubiquitination, sumoylation, poly-ADP ribosylation) that could cause the molecular weight shift and aid the rab17-syntaxin 2 interaction.

P1244

Board Number: B583

Rab35 limits glioblastoma growth in vivo.

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The small GTPase Rab35 is localized to the plasma membrane, clathrin-coated pits and vesicles and endosomes, and mediates the recycling of numerous protein cargos. For example Rab35 is required for the recycling of cadherins, and loss of Rab35 function leads to decreased levels of cadherins on the plasma membrane and loss of cell-cell contacts. In addition, Rab35 recruits its effector ACAP2, a GTPase activating protein for Arf6, to endosomes. Thus, knockdown of Rab35 leads to increased Arf6 activity, which subsequently allows for increased recycling of integrins and enhanced cell migration. Activation of Arf6 following Rab35 knockdown also causes increased cell proliferation through enhanced EGFR recycling and signaling. Given that Rab35 silencing induces decreased cell adhesion and increased cell migration and proliferation, all well known features of invasive cancer cells, we sought to explore the function of Rab35 in cancer. We find that Rab35 mRNA levels are down regulated in surgically resected gliomas compared to normal brain tissues, with the most notable decrease in glioblastoma, the most common and malignant brain cancer. We thus used lentivirus to induce stable knockdown of Rab35 in human glioblastoma-derived brain tumor initiating cells (BTICs), which drive brain tumor growth when transplanted into host mice. Importantly, implantation of Rab35-silenced BTICs resulted in a massive increase in tumor size compared to non-silenced cells. This situation was correlated with a dramatic decrease in survival. Conversely, implantation of Rab35-overexpressing BTICs resulted in a robust decrease in tumor growth with significantly enhanced survival. Thus, Rab35 is a critical factor in controlling the growth of glioblastoma and provides a possible new target for therapy for this devastating brain cancer.

P1245**Board Number: B584****Nitric oxide regulates phagocytosis by S-nitrosylation of Rab5.**M. Hagiwara¹, N. Ishida¹, J. Wang^{1,2}, K. Matsushita^{1,2};¹National Center for Geriatrics and Gerontology, Obu, Aichi, Japan, ²Oral Disease Research, National Center for Geriatrics and Gerontology, Nagoya, Japan

Nitric oxide (NO), produced from L-arginine by nitric oxide synthases (NOSs) in cells, regulates post-translational proteins. Recent studies have been shown that covalent adduction of an NO moiety (NO•) to cysteines, called S-nitrosylation, is a key NO signaling pathway and that regulates protein functions. On the other hand, the small GTPase Rab5 is well known as a crucial protein for endocytosis; however, the mechanism by which Rab5 regulates endocytosis is not known. Here, we report that NO regulates phagocytosis by S-nitrosylation of Rab5. To confirm the effect of NO on phagocytosis, we treated RAW264 cells with an NO donor, GSNO. Phagocytosis was facilitated in RAW264 cells by treatment with GSNO. Inducible NOS (iNOS) was interacted with Rab5 in lysates of HA-Rab5-transfected and then LPS-stimulated Raw264 cells. We next tested the effect of NO on Rab5 activity. Results of a R5BD pull-down assay showed that Rab5 activity was enhanced by treatment with GSNO. Finally, we examined S-nitrosylation of Rab5 by using the biotin switch method. S-nitrosylation was observed more strongly in Rab5Q79L (recombinant, active) than in Rab5S34N (recombinant, inactive). Collectively, our data suggest a mechanism by which NO activates Rab5 and phagocytosis by S-nitrosylation.

P1246**Board Number: B585****Molecular supervisors of Golgi size control.**M.D. Bhave¹, B. Jain¹, P. Iyer¹, D. Bhattacharyya^{2,3};¹Bhattacharyya Lab, ACTREC, Kharghar, Navi Mumbai, India, ²Tata Memorial Centre, Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Kharghar, Navi Mumbai, India, ³Neurobiology, University of Chicago, Chicago, IL

Regulation of size and shape of intracellular organelles is a fundamental yet poorly understood phenomenon in eucaryotic cell biology. We are specifically interested in studying the regulation of size and shape of the Golgi apparatus in the budding yeast *S. cerevisiae*. From our previous study we have shown that ARF1 plays an important role in Golgi size regulation by altering cisternal maturation kinetics[1]. We are currently doing an extensive screening of other Golgi related important proteins for their potential roles in Golgi size regulation. We have deleted various Golgi associated GTPases as well as vacuolar protein sorting genes & studied their effect on size of the Golgi apparatus in *S. Cerevisiae*. Preliminary analysis of knock-out strains has revealed the importance of several GTPases & vacuolar protein sorting genes like Ypt6, Ypt11, Vps21, Vps74, Arl3 in controlling the Golgi size. Systematic study

of these targeted knock outs further will lead to detailed understanding of how these factors play role to control Golgi size.

References: 1. Bhave M, Papanikou E, Iyer P, Pandya K, Jain BK, Ganguly A, Sharma C, Pawar K, Austin J 2nd, Day KJ, Rossanese OW, Glick BS and Bhattacharyya D. Golgi enlargement in Arf-depleted yeast cells is due to altered dynamics of cisternal maturation. J Cell Sci, 2014. 127(Pt 1): p. 250-7.

Establishment and Maintenance of Polarity 1

P1247

Board Number: B587

Nck Regulate Endothelial Cell Morphogenesis by Modulating the Activity of Cdc42.

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Scaffold and adaptor proteins specify the flow of information from activated receptors at the plasma membrane by coordinating the spatiotemporal assembly of intracellular signaling complexes. Here we demonstrate that Nck, an adaptor linking tyrosine phosphorylation with cytoskeletal remodeling, plays an important role in the orchestration of endothelial cell morphogenesis. Using a combination of three-dimensional cultures in collagen matrices, molecular genetics and microscopy, we show that Nck silencing impairs the ability of endothelial cells to self-organize into an interconnected network of tubular structures. Endothelial cells with abrogation of Nck signaling exhibited poor morphogenesis as a result of a reduction in extracellular matrix remodeling and chemotactic invasion. In addition, loss of Nck led to altered organization of the actin cytoskeleton and VE-cadherin-mediated cell-cell junctions. Control and Nck-rescued cells assembled into polarized tubular structures, as evidenced by the presence of patent lumens and apical localization of the marker podocalyxin. In contrast, Nck silenced cells failed to adopt a polarized luminal organization and accumulated vacuoles in their cytosol. Mechanistically, abrogation of Nck signaling impaired the spatiotemporal activation of Cdc42. Experiments to determine the role of Nck in the subcellular localization and activation of polarity pathways, including Par3/Par6/aPKC complex, are underway. Our results suggest an important role for Nck adaptors in the orchestration of the endothelial cell morphogenesis through a mechanism that involves modulation of polarity pathways.

P1248**Board Number: B588****Atypical PKC is dispensable for the maintenance of polarity in differentiated enterocytes. Lessons from the conditional Prkci knockout mouse.**R. Forteza¹, A. Mashukova², Y. Figueroa¹, P.J. Salas^{1,3};¹Department of Cell Biology, University of Miami Miller School of Medicine, Miami, FL, ²College of Medical Sciences, Nova Southeastern University, Fort Lauderdale, FL, ³Univ Miami Sch Med, Miami, FL

The polarity complex, originally described in *C. elegans*, comprises atypical PKC (aPKC) and scaffolding proteins (PARTition deficient) Par6 and Par3. In vertebrates, there are two aPKC isoforms: iota/lambda and zeta. These proteins are highly homologous but encoded by two different genes. They differ in their tissue specific expression. The PKC lambda knock out mouse is embryonic lethal, while the PKC zeta KO is viable and displays only a minor immunologic phenotype. We have shown before that PKC lambda is the major aPKC in intestinal epithelia. In addition, aPKC is deeply post-translationally downregulated in intestinal inflammation. Accordingly we wanted to understand the role of this kinase, considered to be key for polarity, in intestinal epithelia polarity and permeability. We developed a conditional knockout mouse in the C57BL/6 background with Flox sites flanking Exon 4 of Prkci (PKC iota/lambda, ID 18759). After backcrossing with the wild-type to randomize the genetic background, we crossed the animals with Villin-CRE mice. The phenotype was assessed using antibodies against the aPKC phosphorylated turn domain (active conformation) which is totally homologous in both lambda and zeta isoforms, and antibodies against peptides specific for each isoform. The Villin-Cre +;Prkci(flox/flox) offspring were born at the predicted mendelian proportions. These mice were found to have no detectable aPKC signal in the intestinal villus and the colon surface epithelia. Surprisingly, there was a diminished but clear phospho-turn domain signal within the deepest regions of the intestinal crypts. PKC zeta was upregulated specifically in that location and localized to the apical domain, just as PKC lambda in the littermate controls. PKC zeta signal was negative in the distal part of the crypts and the villus epithelium. Despite the lack of aPKC in the absorptive epithelium, the animals gained weight like the controls. There were no differences in 3 kDa fluorescent dextran intestinal permeability. Only 3/26 conditional PKC lambda ko mice showed spontaneous colitis at or before 6 months of age. Membrane polarity markers showed normal polarization. The tight junction marker ZO-1 was also normal, as well as the distribution of Par3. Localization and ultrastructure of TJ were indistinguishable from controls in TEM preparations. Bearing in mind that the life span of epithelial cells in the intestine from the mid-crypt to the tip of the villus is approximately 3 days, we conclude that aPKC is dispensable for the *maintenance* of apical polarity during that period of time. This is not surprising, because similar observations have been made in *Drosophila*. However, these results suggest a need to reconsider the interpretation of the role of aPKC in inflammatory processes and other phenomena involving rapid downregulation of aPKC.

P1249**Board Number: B589****CARACTERISATION OF CERES : A PROTEIN AT THE EDGE OF EPITHELIAL POLARITY AND ENERGY CONTROL.**E. Hardy¹, P. Laprise¹;¹BMBMP, Centre de Recherche sur le Cancer de l'Université Laval - CRCHU de Québec, Québec, QC

Epithelial cell polarization leads to the regionalization of the plasma membrane into apical, lateral and basal domains. The polarized architecture of epithelial cells is crucial for their functions, and also shapes tissues and organs during development. The protein machinery establishing epithelial polarity is conserved from flies to humans, reflecting its significance for the physiology of metazoans. We discovered that the protein Yurt (Yrt) is an evolutionarily conserved regulator of epithelial cell polarity. Yrt plays a critical role by limiting the activity of two master apical determinants, namely Crumbs (Crb) and atypical Protein Kinase C (aPKC). Thereby, Yrt preserves the identity of the lateral membrane and the functional architecture of epithelial cells. Understanding how Yrt acts is instrumental to illuminate the complex network that establishes and maintains epithelial polarity. Towards that goal, we performed a biochemical analysis of the Yrt interactome using mass spectrometry. Our objective is now to characterize the protein Ceres that emerged as a Yrt binding partner.

We produced anti-Ceres antibodies, and confirmed the physical interaction between endogenous Yrt and Ceres by standard co-immunoprecipitation. Ceres expression is dynamic throughout embryogenesis, with a transient accumulation during organogenesis. This suggests that Ceres contributes to epithelial tissue morphogenesis. Ceres subcellular distribution is mainly cytoplasmic, and this protein shows a punctate distribution that likely reflects an association with intracellular vesicles. To investigate Ceres function, we generate a null allele using the CRISPR/Cas9 system. *ceres* mutant embryos do not display polarity defects. However, loss of Ceres rescues the *yrt* mutant embryonic phenotype. This argues that Ceres antagonizes Yrt activity to fine-tune polarity regulation. Further analysis of the *ceres* mutant phenotype revealed that newly hatched mutant females produce fertile eggs, whereas ovarian follicles degenerate at stage 8 of oogenesis in older flies. A similar phenotype is encountered in starved wild type females. This suggests that flies devoid of Ceres are under a metabolic stress. In support of this hypothesis, *ceres* mutant animals show a higher activation level of the AMP-activated protein kinase when compared to their wild type counterparts.

Overall, our data identify a novel putative regulator of Yrt activity and epithelial cell polarity, which is crucial for animal development and physiology. In parallel, we established that Ceres controls the cellular energetic balance, suggesting that Yrt and other polarity regulators may also play a role in this process. Thus, our data highlight a possible link between polarity regulation and metabolism.

P1250**Board Number: B590****PAR3 and aPKC regulate Golgi organization through CLASP2 phosphorylation for cell polarity.**T. MATSUI¹, T. Watanabe¹, K. Kaibuchi¹;¹Nagoya University School of Medicine, Nagoya, Japan

The organization of Golgi apparatus is essential for cell polarization and its maintenance. A PAR complex, composed of PAR3, PAR6 and aPKC (atypical PKC), plays a central role in establishment of cell polarity through the several cellular processes. However, it remains largely unknown how the PAR complex participates in the regulation of the Golgi organization. By screening for Par3-associating candidates, we previously identified another polarity protein CLASP2, which controls Golgi ribbon organization for front-rear polarity in migrating cells. Focusing on CLASP2, we demonstrate a novel molecular mechanism of the PAR complex in the regulation of the Golgi apparatus. We found that PAR3 formed a complex with CLASP2 through direct interaction. Similar to the case of other PAR3-binding proteins, CLASP2 was phosphorylated by aPKC. In epithelial cells, knockdown of either PAR3 or aPKC induced the aberrant CLASP2 accumulation at the trans-Golgi network (TGN) concomitantly with disruption of the Golgi ribbon organization. The expression of the CLASP2 mutant, which inhibited the PAR3-CLASP2 interaction, disrupted the Golgi ribbon organization. CLASP2 is known to localize to the TGN through its interaction with the TGN protein GCC185. This interaction was impaired by PAR3- and aPKC-mediated phosphorylation of CLASP2. Furthermore, the non-phosphorylatable mutations enhanced the co-localization CLASP2 with GCC185, thereby impairing the Golgi organization. Based on these observations, we propose that PAR3 and aPKC control the Golgi organization by regulating CLASP2 binding ability to GCC185.

P1251**Board Number: B591****Par3 limits APP and BACE1 convergence by directing their trafficking to two distinct pathways.**M. Sun¹, H. Zhang¹;¹Department of Neuroscience and Cell Biology, Rutgers University, Piscataway, NJ

The aging process is intricately linked to cell polarity. Loss of cell polarity has been implicated in different age-related disorders such as cancer and stem cell aging. However whether brain aging represents a polarity defect is not known. Alzheimer's disease (AD) is the most common form of neurodegenerative diseases affecting the elderly. It is characterized by extracellular plaques made up of β -amyloid peptides (A β), which is derived from the transmembrane protein APP through cleavage by β - and γ -secretases. Previous studies suggest the intracellular trafficking properties of APP and its secretases determine how APP is processed, with amyloidogenic processing occurring mostly in endocytic compartments and non-

amyloidogenic processing occurring on the cell surface. However, how the trafficking properties of APP and its secretases are regulated is still not well understood. The Par polarity complex is emerging as a key regulator of vesicle trafficking. This complex consists of Par3, Par6 and atypical PKC (aPKC), which are conserved proteins that regulate cell polarization from worms to mammals. Here we show that loss of the polarity protein Par3 is associated with Alzheimer's disease pathogenesis. Knockdown of Par3 promotes amyloidogenic APP processing and increases A β generation, while overexpression of Par3 promotes non-amyloidogenic APP processing. We further show that Par3 regulates APP trafficking by promoting its targeting to the recycling pathway, while knockdown of Par3 leads to lysosomal targeting of APP. Unexpectedly, we found that Par3 also regulates the trafficking of the β -secretase BACE1. However, loss of Par3 does not seem to cause a global disruption of vesicular trafficking. Rather, Par3 regulates APP and BACE1 trafficking through two distinct mechanisms. While Par3 regulates APP trafficking through the small GTPase Rac, it promotes BACE1 retrograde trafficking to the TGN by recruiting aPKC, which phosphorylates BACE1 on the C-terminus. Finally, we found that Par3 protein level is decreased in both human AD brain samples and a mouse AD model, and treatment of neurons with A β leads to a decrease in Par3. This suggests that there exists a vicious cycle in the AD brain where A β leads to a decrease in Par3, which then causes APP and BACE1 convergence in the lysosomes, leading to a further increase in A β generation. Taken together, our studies reveal a novel role for the polarity protein Par3 in AD pathogenesis through a two-pronged mechanism that involves regulation of APP and BACE1 trafficking.

P1252

Board Number: B592

Mechanochemical feedback regulates the dynamics of the PAR system in *C. elegans* zygotes.

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The interplay between regulatory biochemistry and cell mechanics is critical for a broad range of morphogenetic changes. Cell mechanics can induce transport via growth and flow-fields, which in turn affect concentration-fields of regulators. Such systems exhibit an intrinsic feedback-architecture between regulators of cell mechanics and mechanical deformation. While we anticipate that this feedback between biochemistry and cell mechanics is widespread in Morphogenesis, there are few examples that are studied with respect to their potential for generating spatiotemporal patterns.

Here we establish at a quantitative level that PAR polarization of *C. elegans* zygotes represents a coupled mechanochemical system. Using Fluorescence Recovery After Photobleaching (FRAP) and RNA interference (RNAi), we first demonstrate that the biochemistry in form of the PAR domains feeds back on the mechanics by establishing and maintaining a non-muscle myosin II (nmy-2) gradient. Additionally,

we characterize the effect of the polarity cue associated with the centrosome of the male pronucleus on the local myosin concentration at the posterior pole. We show that it induces a reduction in myosin concentration and thereby triggers the onset of cortical flows. Furthermore we measure the spatiotemporal profile of the anterior and posterior PAR concentration, the myosin II concentration and the induced flow-field.

Finally, we capture the feedback-architecture of the coupled actomyosin – PAR system in a quantitative model, based on coupling a thin film active fluid description of cortical mechanics [1] to a reaction-diffusion PAR patterning system [2]. We show that this mathematical model can quantitatively recapitulate the spatiotemporal profile of PAR polarity establishment. Furthermore, we demonstrate that the model predicts the existence of a threshold in cortical flow velocity, which separates the non-polarizing and the polarizing regime and confirm the existence of this threshold velocity in the living *C. elegans* zygote.

Taken together, we show that the spatiotemporal chain of events that constitute PAR polarization in *C. elegans* can be described quantitatively in terms of a coupled, active mechanochemical pattern generating system.

[1] Mayer, M., Depken, M., Bois, J. S., Jülicher, F. & Grill, S. W. Anisotropies in cortical tension reveal the physical basis of polarizing cortical flows. *Nature* **467**, 617–621 (2010).

[2] Goehring, N. W. et al. Polarization of PAR Proteins by Advective Triggering of a Pattern-Forming System. *Science* **334**, 1137–1141 (2011).

P1253

Board Number: B593

An Instructive Role for *C. elegans* HMR-1/E-cadherin in Translating Cell Contact Cues into Cortical Polarity.

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Early embryonic cells in many species polarize radially by distinguishing their contacted and contact-free surfaces. The homophilic adhesion protein E-cadherin is required for contact-induced polarity in many cells. However, it is not clear whether E-cadherin functions instructively as a spatial cue, or permissively by ensuring adequate adhesion so that cells can sense other contact signals. In *C. elegans*, radial polarity begins at the four-cell stage, when cell contacts restrict the PAR polarity proteins to contact-free surfaces. We previously identified the RhoGAP PAC-1 as an upstream regulator that is required to exclude PAR proteins from contacted surfaces of early embryonic cells. PAC-1 is recruited specifically to sites of cell contact and directs PAR protein asymmetries by inhibiting the Rho GTPase CDC-42. How PAC-1 is able to sense where contacts are located and localize to these sites is unknown. We show that HMR-1/E-cadherin, which is dispensable for adhesion, functions together with HMP-1/ α -catenin, JAC-1/p120 catenin, and the previously uncharacterized linker PICC-1/CCDC85/DIPA to bind PAC-1 and

recruit it to contacts. Furthermore, we show that ectopically localizing the intracellular domain of HMR-1/E-cadherin to contact-free surfaces of cells recruits PAC-1 and depolarizes cells, demonstrating that HMR-1/E-cadherin plays an instructive role in polarization. Our findings identify an E-cadherin-mediated pathway that translates cell contacts into cortical polarity by directly recruiting a symmetry-breaking factor to the adjacent cortex.

P1254

Board Number: B594

The effects of PAM-1 suppressors on the polarization of *C. elegans* embryos.

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In *Caenorhabditis elegans*, anterior-posterior axis formation begins shortly after fertilization. The goal of this project was to understand this process through the study of *pam-1*, a gene involved in polarization. When *pam-1* is compromised, the embryo fails to polarize and as a result, the embryo dies. This is due to the lack of the puromycin-sensitive aminopeptidase PAM-1 that is coded for by this gene. Without PAM-1 to initiate polarization, the embryo does not properly form an anterior-posterior axis. We study *pam-1* by isolating mutants that rescue the *pam-1* phenotype, called suppressors. We cross strains containing the *pam-1* suppressor and hatch tests are performed to determine if the mutations still exist. Once verified, genomic mapping is performed to identify the suppressor's chromosomal location. This mapping will allow us to identify each suppressor gene and understand how it interacts with *pam-1*. Another way we study *pam-1* is by observing the polarization of the one-celled embryo using both fluorescent proteins under a confocal microscope and differential interference contrast (DIC) microscopy. Using these techniques, time-lapse images of the single cell dividing into a two-celled embryo are recorded. Later, videos are analyzed and the time at which certain landmarks of anterior-posterior axis formation occur is noted and compared in suppressor strains. Knowing more about related genes will help us to better understand polarization. From our studies, we have isolated two strains, *lz2* and *lz5*, that suppress the lethality of *pam-1*. The suppressors have been outcrossed; the hatch rates of the mutant strains and rescue of polarity, as evidenced using DIC microscopy, have led to the conclusion that the suppressors display a semi-dominant phenotype. Progress has been made using SNP mapping of the suppressors to determine the chromosomal location of the suppressor mutation. With further tests and observations to characterize these strains, the targets of PAM-1 and its role in biological pathways can be further elucidated.

P1255**Board Number: B595****The Identification and Characterization of pam-1 Targets Through the Use of Suppressors in *Caenorhabditis elegans*.**T. Nguyen¹, A. Brewer¹, M. Kurland¹, S. Shah¹, E. Jaeger¹, R.L. Lyczak¹;¹Biology, Ursinus College, Collegeville, PA

Within the one cell embryo of *Caenorhabditis elegans*, cell polarity is established before the first cell division. A crucial part of this process involves the *pam-1* gene of the organism, which codes for a puromycin-sensitive aminopeptidase, or PAM-1 protein. The PAM-1 proteins are conserved in plants and animals, have a range of different functions which includes protein degradation, protein maturation, and aiding in protein stability and may play a role in neurodegenerative diseases such as Huntington's and Alzheimer's disease. PAM-1 plays an important role in the positioning of the centrosome in *C. elegans* embryos which allows them to establish the anterior-posterior (AP) axis and develop normally. Our study focused on the mutated *pam-1* gene. Embryos with this mutation demonstrate an embryo-lethality effect due to the failure to form the anterior-posterior (AP) axis. In order to identify regulators and targets of PAM-1, we identified suppressors, mutations that reverse the lethality effect. We worked with two suppressors, *lz3* and *lz4*, that suppress the lethality of a missense allele of *pam-1*. The goal was to genetically map the chromosomal location of *lz3* and *lz4* using single nucleotide polymorphisms (SNP) mapping. Strains of progenies of a genetic cross of the suppressor and the wild-type strain were validated for SNP mapping, and chromosome I or IV were identified to be the possible locations of *lz3* and *lz4*. Overall, we hope to characterize all the current suppressors of mutated *pam-1* and their genotypes in addition to verifying new, potential suppressors. The use of suppressor screens can also help identify other proteins involved in the processes regulated by PAM-1.

P1256**Board Number: B596****Role of centrosome in anterior-posterior axis formation in single-celled *Caenorhabditis elegans* embryos.**D.M. Saturno¹, D. Parikh¹, D. Stephens¹, D. Castanzo¹, M. Williams¹, R.L. Lyczak¹;¹Biology, Ursinus College, Collegeville, PA

The formation of the anterior-posterior (AP) axis in single-celled *Caenorhabditis elegans* embryos is crucial to achieving functional potential. The entrance of sperm into the oocyte triggers completion of meiosis and initiates polarity establishment. In wild-type (WT) cells, the protein PAR-6 is initially evenly distributed along the inner boundaries of the embryo, however interactions between the centrosome and posterior cortex prompt PAR-6 to accumulate at the future anterior end, indicating successful polarization. *pam-1* mutants lack an active puromycin-sensitive aminopeptidase (PAM-1) protein. In these embryos, which fail to completely polarize, PAR-6 demonstrates an inability to localize. Our

research seeks to determine the differences between *pam-1* mutants and WT embryos in the progress of polarity establishment, specifically centrosome contact with the posterior cortex. Time-lapse confocal microscopy was used to study the movement of GFP-tagged centrosomes and chromosomes in the WT and *pam-1* strains as the embryo polarizes. We found that in WT strains, the centrosomes interact with the cortex for about four minutes during the early pronuclear stage. In *pam-1* strains, the centrosome interacts with the cortex earlier in the cell cycle and for a shorter duration. Delayed meiotic exit in *pam-1* mutants, in addition to shorter centrosome-cortex contact, supports the idea that the centrosome must maintain contact with the cortex in order to sustain the polarization process. Our results suggest that both timing and duration of centrosome contact must be regulated to ensure axis polarization. PAM-1 is necessary for proper centrosome positioning.

P1257

Board Number: B597

Cadherin-Integrin independent epithelium formation depends upon ZEN-4/MKLP in *C. elegans*.

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How are polarized epithelia established and maintained? This question is of critical importance, as the loss of epithelial polarity is associated with human diseases, such as metastasis. The current paradigm is that adhesive signals, either at the junctional domain or at the basal surface, provide spatial cues that properly polarize the cell. Recent studies, however, have challenged this notion, suggesting the existence of a poorly-understood non-canonical polarity pathway. I have now shown that epithelial polarity in *C. elegans* is independent of the traditional 'outside-in' cues, making this an ideal system to identify factors involved in this alternative pathway.

We have begun an analysis of non-canonical epithelium formation using the *C. elegans* foregut. The nine arcade cells undergo a mesenchymal-to-epithelial transition (MET) at mid-embryogenesis, linking the foregut to the epidermis. The Mango lab performed an unbiased screen for defects in arcade cell MET. This screen revealed that ZEN-4/MKLP1 is essential for arcade cell polarization. In *zen-4* mutant arcade cells, markers of the apical and junctional domains are absent. Interestingly, my data indicate that ZEN-4 acts at the time of arcade cell birth rather than during polarization. This means that ZEN-4 is acting in the arcade cells ~100 minutes prior to the onset of any visible signs of polarity (e.g. PAR-6, E-cadherin). To elucidate the role of ZEN-4 in polarity, we performed structure-function analysis of this mitotic kinesin, which is thought to migrate toward microtubule plus ends and to bundle antiparallel microtubules to generate the central spindle during cytokinesis. Our studies revealed that the motor functions of this kinesin are dispensable for polarity, but not conserved residues important for binding its obligate partner in cytokinesis, CYK-4/GAP. How does this complex function in polarity? We focused on a conserved regulator of polarity, PAR-6/PDZ. In *par-6* mutants, apicobasal markers are detectable in arcade cells but not localized properly. Our model is that *zen-4* is required for PAR-6 expression in

arcade cells, which is then required to ensure proper localization of apical and junctional proteins. Our current experiments are testing this model.

P1258

Board Number: B600

Attenuation of N-glycosylation causes polarity and adhesion defects in the *C. elegans* embryo.

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The one-cell stage *C. elegans* embryo is highly polarized, requiring sequestration of cytoplasmic polarity factors at the plasma membrane and compartmentalization of the plasma membrane itself. Compartmentalization would aid asymmetric distribution of lipids and proteins, being partially responsible for the fates of the future daughter cells. Since most plasma membrane proteins are glycosylated, we determined the effect of reduction of N-glycosylation on early development. We show that the polarity factor PAR-2 requires at least one N-glycosylated protein for efficient recruitment to the posterior cortex. More importantly, cell-cell adhesion was specifically lost only at the two-cell stage upon reduction of N-glycosylation. This loss-of-adhesion phenotype was rescued by interfering with polarity establishment, indicating that polarity establishment is upstream of plasma membrane segregation. Remarkably the epithelial adhesion complex components E-cadherin and MAGI-1 were found at contact sites at the two cell stage embryos. This localization was lost under reduced N-glycosylation, but was rescued by concomitant loss of PAR-2.

P1259

Board Number: B601

Regulation of apical-basal polarity during epithelial tissue regeneration.

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Almost all organisms have the capacity for some tissues to regenerate at certain times in development. However, little is known about the morphogenesis of regenerative tissues. In particular, how is established tissue architecture remodeled to incorporate regenerated tissues? In *Drosophila* larvae, imaginal discs – larval precursors to adult appendages – have substantial regenerative capacity. Imaginal discs are composed of a primary layer of epithelial cells and a secondary squamous epithelia. This simple structure has allowed us to study changes in epithelial tissue architecture during regeneration.

We have observed a transient loss of apical-basal polarity (ABP) during imaginal disc regeneration. Using two common markers for maintenance of cellular ABP, atypical protein kinase C (aPKC) and Discs large (Dlg), we observe disruption of apical and apical-lateral staining in a subset of cells within the

regenerating disc after tissue damage induced by irradiation. This disruption of ABP is seen when dying cells are just beginning to migrate basally, and persists for ~24 hours after damage is induced. This regenerative epithelia remodeling is reminiscent of neoplastic transformation in imaginal disc tumor models, suggesting that these may be mechanistically related processes. However, in contrast to neoplastic transformation, ABP disruption during regeneration appears to be quickly remodeled, with restoration of ABP taking place concurrent with the extrusion of dying cells from the imaginal disc epithelia. Within ~ 36 hours, polarity is restored; residual/extruded caspase positive cells remain present for almost ~ 48 hours following irradiation. Regions of polarity loss appear to include both dying and living cells, leading us to examine whether dying cells are capable of producing a non-autonomous polarity change in surrounding cells.

To this end, we are examining the *wingless* and JNK signaling pathways, which have been implicated in compensatory responses to tissue damage, to determine if these pathways function in reorganization of tissue polarity during the regenerative response.

Understanding how ABP is regulated following damage will provide new insights into regenerative morphogenesis, and may reveal pathways that regulate the loss of epithelial tissues architecture during tumorigenesis.

P1260

Board Number: B602

The *Drosophila* RhoGEF Cysts is essential for epithelial polarity.

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The function of RhoGEFs (Rho GTPase-specific guanine nucleotide exchange factor) is critical for development and reducing the risk of cancer. RhoGEFs promote the cyclic activation and localization of small GTPases like Rho, Rac, and Cdc42, which promote the apicobasal organization of epithelial cells through the dynamic regulation of polarity and adhesion complexes. How a wide diversity of RhoGEFs coordinate the activity of a limited number of small GTPases remains unclear. The purpose of this project is to characterize a novel RhoGEF in *Drosophila* that we named Cysts. Cysts is the single *Drosophila* orthologue of a group of four mammalian RhoGEFs (p114RhoGEF, p190RhoGEF, AKAP-13, and Lsc/GEF-H1). Using a maternal shRNA targeting approach we have found that Cysts is required for epithelial polarity. The loss of Cysts causes a disruption of adherens junctions and multi-layering by embryogenesis, followed by apoptosis of many epithelial cells. Depletion of Cysts also leads to the apical constriction and aberrant cortical enrichment of actin in small groups of cells, which go on to form cyst-like clusters (hence the gene name *cysts*), similar as seen in *crumbs* mutant embryos or embryos compromised for other polarity proteins. Our preliminary evidence suggests that aPKC is a downstream target of Cysts. Further, we have found that a Cysts::GFP fusion protein is apico-cortically enriched. Structure-function analysis of Cysts reveals that the DH-PH (RhoGEF, pleckstrin-homology) domain is

required for Cysts function, and that the N-terminal region is required for cortical localization. These results implicate Cysts as a novel RhoGEF that is required for the integrity of epithelial polarity in *Drosophila*. We are currently working to define the GTPase target of Cysts and generate a null mutant for *cysts* using CRISPR technology.

P1261

Board Number: B603

Regulation of planar cell polarity through Dachs-Sple and Ds-Sple interaction in *Drosophila melanogaster*.

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Planar cell polarity (PCP) is the polarization of cells in the plane of a tissue, orthogonal to their apical–basal axis. Two molecular systems are involved in establishment and maintenance of PCP: Frizzled PCP and Ds-Fat PCP. The Frizzled system regulates PCP locally whereas in Ds-Fat system, there are long-range patterning cues in the form of protein gradients, which orient this polarization with the axes of the tissue.

In the Ds-Fat PCP pathway, the receptor Fat (transmembrane cadherin protein) is activated upon binding to its ligand Dachsous (Ds) from neighboring cells. Four-jointed (Fj) is a Golgi-localized kinase that phosphorylates cadherin domains of Fat and Ds to modulate binding between them. Ds and Fj are expressed in opposing gradients, which influence Fat activity and result in polarization of the downstream effector Dachs (unconventional myosin) along the direction of these gradients. In the Frizzled PCP pathway, the membrane spanning protein Frizzled (Fz) on distal side of the cell forms heterotypic interaction with another membrane spanning protein Strabismus (Stbm) on proximal side of the cell. Flamingo (Fmi) is present on both the sides of cell and interacts homotypically. The cytoplasmic proteins Dishevelled (Dsh) and Diego (Dgo) are associated with Fz-Fmi complex whereas Prickle (Pk) is associated with the Stbm-Fmi complex. Prickle is present in two isoforms: Prickle (Pk) and Spiny-legs (Sple) and balance between these isoforms is crucial for normal PCP. Loss of a single isoform (Pk or Sple) results in more severe hair polarity phenotype than that caused by loss of both the isoforms (Pk-Sple). These cytoplasmic components interact with downstream tissue specific effectors to direct PCP.

Although the molecular mechanisms by which the Frizzled pathway mediate local polarity are known, it was not clear how this pathway interacts with Ds-Fat pathway to coordinate polarity along the axis of a tissue. Recently, it has been reported that Sple-Dachs interaction can polarize Sple along the gradient of Ds in wing disc. Here, we show that Sple can physically bind to both Dachs and Ds and its N-terminal domain is sufficient for this interaction. Loss of Dachs results in reversal of Sple polarity in wing disc and can suppress *pk* mutant hair polarity phenotype in wing. These observations indicate that the Ds-Fat pathway might influence the balance between Pk and Sple localization through Sple-Dachs or Sple-Ds interaction. Moreover, Sple is polarized in the same direction as Dachs in tarsal segments of the leg and abdomen, indicating that Sple-Dachs or Sple-Ds interaction can regulate PCP in these tissues as well.

Hence, this would provide a global mechanism for directional input from the Ds-Fat pathway to the Frizzled pathway.

P1262

Board Number: B604

The small GTPase Rac1 controls tubulogenesis in *Drosophila melanogaster*.

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The plasma membrane of epithelial cells is segregated into an apical, a lateral and a basal domain. This polarization is orchestrated by several molecular modules that interact with each other to fine tune their respective activity, thereby ensuring the physiology and homeostasis of epithelial tissues. Failure of this crosstalk has been linked to morphogenesis defects. Epithelia can fold into tubes to build complex structures like the branched network of airways. However, how the establishment of such a three-dimensional architecture is coordinated at the cellular and subcellular levels remains elusive. To illuminate these issues, we use the *Drosophila* trachea as model. This tissue undergoes several morphogenetic processes to reach its correct diameter and length. These processes are controlled by an apical matrix pathway that requires functional septate junctions and secretion of the chitin-modifying enzymes Verm and Serp in the tracheal lumen. In addition, the Crb epithelial polarity module promotes apical membrane growth in tracheal cells, thereby specifying tube size. Consequently, overexpression of Crb results in an abnormally long trachea. Inhibition of Cora or Yrt modules, which antagonize Crb, leads to a comparable phenotype. Similar to Cora and Yrt, the GTPase Rac1 has been described to establish a reciprocally antagonistic relationship with Crb in the *Drosophila* ectoderm, suggesting that it may control epithelial tube morphogenesis.

Here, we show that expression of a dominant negative form of Rac1 in tracheal cells increases tube length without changing cell number. This phenotype is rescued by reducing *crb* dosage. This demonstrates that epithelial tube overgrowth results from an elevation of Crb activity, and suggests that Rac1 normally represses Crb activity to control tracheal tube length. Using genetic interactions, we established that Rac1 acts in parallel to *yrt* to control Crb activity. In addition, we observed decreased Rac1 activity in *cora* mutant or Cora knockdown embryos. This suggests that Rac1 is an effector of Cora regulating Crb activity and epithelial tissue morphogenesis. Accordingly, we found that Rac1 activity promotes secretion of Verm and Serp and septate junction integrity, as reported for Cora.

Overall, our data establish that Rac1 activity is required for proper tubulogenesis. It likely mediates Cora-dependent inhibition of Crb and apical membrane growth. In addition, Rac1 participates in the apical matrix pathway. Further characterization of these molecular mechanisms will allow for a better understanding of animal development and diseases associated with tube size defects.

P1263**Board Number: B605****The Balbiani body proteome in zebrafish.**A. Jamieson-Lucy¹, M.C. Mullins¹;¹Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA

The Balbiani body is the first marker of polarity in vertebrate oocytes and presents a novel mechanism generating cell polarity. The Balbiani body is a conserved structure found in diverse animals including insects, fish, amphibians, and mammals. During early zebrafish oogenesis, the Balbiani body assembles as a transient aggregate of mRNA, proteins, and membrane-bound organelles at the presumptive vegetal side of the oocyte. As the early oocyte develops, the Balbiani body translocates from its initial perinuclear position to the vegetal cortex, where it disassembles and deposits its cargo of localized mRNAs and proteins. In fish and frogs, this cargo includes the germ plasm as well as gene products required to specify dorsal tissues of the future embryo. Despite its central role in establishing oocyte polarity, little is known about the mechanism behind the Balbiani body's action. Analysis of the few known protein components of the Balbiani body is insufficient to explain how the Balbiani body assembles, translocates, and disassembles. We isolated Balbiani bodies from zebrafish oocytes and performed mass spectrometry to define the Balbiani body proteome. We successfully identified 150 proteins associated with the Balbiani body sample, including known Balbiani body proteins and novel RNA-binding proteins. These results are a prerequisite to a comprehensive mechanism for Balbiani body function at a protein level. Furthermore, the number of proteins identified indicates that the Balbiani body is a complex structure that requires many proteins working in concert to generate oocyte polarity.

P1264**Board Number: B606****microtubule-actin crosslinking factor (Macf1) Functions in Animal-Vegetal Polarity Establishment In Zebrafish Oocytes.**M. Escobar-Aguirre¹, A. Jamieson-Lucy¹, R. Fuentes¹, M.C. Mullins¹;¹Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA

In zebrafish oocytes, animal-vegetal polarity (AV) is established early in oogenesis through formation and translocation of the Balbiani Body (Bb) to the prospective vegetal pole of the oocyte where it disassembles at the end of stage I oogenesis. The Bb is a highly conserved structure present from insects to humans that contains mitochondria, ER, RNAs and proteins. Our lab demonstrated that the establishment of AV polarity depends on the function of the bucky ball and macf1 genes, which are required for Bb formation and disassembly, respectively. Loss of Macf1 function causes: 1) an enlarged Bb 2) asymmetric nucleus and 3) failure in Bb disassembly. Macf1 is a spectraplakins that connects actin, intermediate-filaments (IFs) and microtubules (MT) to functionally integrate them. Our aim is to elucidate the Macf1 dependent mechanism that -through interaction with the cytoskeleton- functions in

AV polarity establishment in oocytes. First, we determined that Macf1 protein localizes to the Bb and upon disassembly distributes along the vegetal cortex like Bb-localized mRNAs and other Bb components. Then, we examined the distribution of cytoskeleton components and assessed their function in Bb structure and nuclear positioning. To accurately determine the cytoskeleton distribution throughout stage I of oogenesis, we developed a MATLAB-based method to stage early oocytes. The algorithm accounts for oocyte size and Bb position to precisely determine the developmental stage of the oocytes. In early oocytes, Cytokeratins (CK) show a perinuclear distribution, and as stage I progresses CK distribute in the cytoplasm and accumulate in the Bb. In *macf1* mutants, CK enrichment in the Bb is greatly diminished. On the other hand, MT do not associate with the Bb and disruption of MT does not affect Bb structure or nuclear positioning. In *macf1* mutants, the asymmetric nuclear position coincides with the onset of the Bb enlargement defect, hence we genetically addressed whether the asymmetric nucleus is secondary to the Bb enlargement by examining *macf1*; *macf1* double mutants. Indeed, double mutants exhibit an asymmetric nuclear position, which demonstrates that Macf1 function in nuclear positioning is independent of Bb formation. In summary, we propose a model where CK maintain Bb structure until it reaches the vegetal cortex. Here, the Bb disassembles by Macf1 function in integrating cortical actin with CK and/or MT in a dynamic process culminating in AV polarity establishment. Ongoing live-imaging experiments will shed light on the Macf1-dependent cell polarity mechanism acting in developing zebrafish oocytes.

P1265

Board Number: B607

A GDI-independent mechanism to concentrate the Rho-GTPase Cdc42 during polarity establishment.

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The highly conserved Rho-family GTPase Cdc42 is a critical regulator of polarity in many fungal and animal cells. GTP-Cdc42 is concentrated in a region of the plasma membrane specifying the “front” of the cell, and interacts with downstream effectors to orient the cytoskeleton along the front-back axis. In the budding yeast, *Saccharomyces cerevisiae*, an autocatalytic mechanism enables resident GTP-Cdc42 to activate nearby GDP-Cdc42 via recruitment of a GDP/GTP exchange factor (GEF), creating a local GTP-Cdc42 enrichment over GDP-Cdc42. Subsequent delivery of more Cdc42 to that site would yield an overall increase in total Cdc42 (GDP+GTP) concentration. A GDP-dissociation inhibitor (GDI), Rdi1, is thought to deliver GDP-Cdc42 from distant parts of the membrane through the cytoplasm to the polarity site, where the GEF converts it to GTP-Cdc42. However, deletion of *RDI1* does not block Cdc42 concentration, so other Cdc42-delivery pathways must exist. One proposed mechanism involves delivery of Cdc42 via actin-directed vesicle trafficking, but we found that Cdc42 accumulated at the polarity site before exocytic vesicles in *rdi1Δ* cells. Furthermore, actin depolymerization did not block concentration of Cdc42 in *rdi1Δ* cells. Confocal imaging revealed a cytoplasmic pool of Cdc42 in *rdi1Δ*

cells, which could be reduced by additional lipid modification of Cdc42. Thus, Cdc42 can still exchange between the cytoplasm and the plasma membrane in *rdi1Δ* cells via an undiscovered parallel pathway, but at a slower rate. To search for components involved in that pathway, we conducted a genetic screen for mutants that are synthetically lethal with deletion of RDI1. We isolated several alleles of BEM2, a Cdc42-directed GTPase activating protein (GAP). Conditional *rdi1 bem2* double mutants arrested as large, round, and unpolarized cells. Our findings suggest that Cdc42 stills traffic through the cytoplasm without the GDI in a parallel pathway, and that cells relying on such traffic unexpectedly require the GAP Bem2.

P1266

Board Number: B608

RGS Proteins and Septins Cooperate to Promote Chemotropism by Restricting Polar Cap Mobility.

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Septins are well known to form a boundary between mother and daughter cells in mitosis, but their role in other morphogenic states is poorly understood. Using microfluidics and live cell microscopy, coupled with new computational methods for image analysis, we have investigated septin function during pheromone-dependent chemotropic growth in yeast. We show that septins colocalize with the regulator of G protein signaling (RGS) Sst2, a GTPase-activating protein that dampens pheromone receptor signaling. We show further that the septin structure surrounds the polar cap, restricting its movement and ensuring that cell growth is directed toward the source of pheromone. Once formed, these structures constitute a dynamic barrier by scaffolding the RGS protein. When RGS activity is abrogated, septins are mislocalized and the polar cap travels toward septin structures and away from sites of exocytosis, resulting in a loss of gradient tracking. Thus septins promote chemotropic growth, and their function is dynamically controlled by RGS proteins.

P1267**Board Number: B609****Role of sterol-rich membrane domains in fission yeast cell polarity establishment and maintenance.**T. Makushok¹, P. Alves², S.M. Huisman³, D. Brunner⁴;¹Biochemistry and Biophysics Dept., University of California, San Francisco, San Francisco, CA, ²IGBMC, Illkirch, France, ³UZH, Zurich, Switzerland, ⁴Institute of Molecular Life Sciences, University of Zürich, Zurich, Switzerland

Fission yeast (*Schizosaccharomyces pombe*) is a model organism that is widely used for studying cell polarization. Polarization in fission yeast involves cytoskeleton-mediated positioning of growth sites. A complex consisting of the polarity factors Tea1 and Tea4 is transported on microtubules to the cell end regions. Tea4 interacts with the actin polymerization promoter For3. Selective activation of For3 in the cell end regions makes them rich in F-actin that contributes to localized cell growth. In this study, sterol-rich membrane (SRM) domains present at the growth sites are introduced as a new element in the picture, and their role in cell polarity establishment is analyzed. Since SRMs are absent from the plasma membrane in starved cells, imaging of cells recovering from starvation using the novel SRM marker GFP-Tna1 was performed to follow SRM domain formation *de novo*. Automated image analysis software was developed to analyze and correlate cell growth and SRM dynamics with an unprecedented level of precision. The results show that properly formed SRM domains are essential for fission yeast growth. SRMs, and with them the growth machinery, have to polarize before cell growth initiation. F-actin is required for selective removal of SRM domains in the cell middle region, and thus for polarizing SRMs. Fast removal of SRM domains in the cell middle region is not due to increased endocytic activity (mediated by F-actin). Tea1 controls the localization of polarized growth via Tea4 by affecting the positioning of SRM domains. Tea1 and Tea4 are essential for the stability of the SRM domains not associated with active growth sites. The importance of the microtubule cytoskeleton for the stability of SRM positioning stems from its role in the transport of the Tea1-Tea4 complex to the cell end regions. *Tea1Δ* and *tea4Δ* cells, known to grow monopolarly, grow faster at individual cell ends than wild type cells. Tea1 and Tea4 are required for proper timing of growth initiation. The proteins associated with the actin cytoskeleton, For3 and its activator Bud6, are important for the stability of SRM domains at both cell ends. For3 is also important for growth speed stabilization. Thus, a complex feedback loop links SRMs and cell growth. SRMs are essential for the polarization of the growth machinery, probably serving as platforms for its recruitment. The growth machinery, in turn, seems to stabilize SRM domains at sites that have initiated growth. The results of this study show that SRMs are a critical factor in *de novo* cell polarization, and not merely a player in its maintenance.

P1268**Board Number: B610****Systematic Analysis of Yeast F-box Proteins Reveals a New Role of Ubiquitination in Polarity Establishment.**J.B. Sheetz¹, J.B. Kelley^{1,2}, S.M. Miller², J.H. Hurst¹, E.G. Young¹, H.G. Dohlman^{1,2};¹Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC, ²Department of Pharmacology, University of North Carolina, Chapel Hill, NC

Ubiquitination is a post-translational modification that regulates protein activity by tagging specific substrates for degradation. Regulation of proteins by ubiquitination has been demonstrated to help terminate the response to mating pheromone in budding yeast. The yeast pheromone response is comprised of two branches, one leading to MAPK activation and gene transcription, the other leading to cellular polarization and shmoo formation. The Skp1/Cdc53/F-box (SCF) ubiquitin ligase employs different F-box subunits to recruit specific substrates for ubiquitination. In yeast, F-box proteins have been identified as interchangeable components of the SCF complex. One of these, Cdc4, specifically targets pathway components including Gpa1, Far1 and Ste7. To identify additional pathway regulators, we conducted a screen in which we measured activation of the pheromone response in 16 yeast strains -- each lacking one of the known (or candidate) F-box genes. To gain an understanding of specific F-box functions, we characterized the abilities of these deletion mutants to affect either the MAPK or the polarity branch of the pathway. Our study reveals that one F-box protein, Pfu1, selectively regulates the polarity branch. Using live cell imaging in a microfluidic chamber, we demonstrate that Pfu1 is required to restrict the cell to a single polarity site. Cells lacking Pfu1 produce multiple polar caps and grow simultaneously during pheromone stimulation. In contrast, Pfu1 has no effect on MAP kinase activation or transcriptional induction. Candidate targets for Pfu1 are being evaluated using protein stability assays and mass-spectrometry sequencing. These findings are significant because they reveal new components of the cell polarization machinery. While polar axis formation is a fundamental and well characterized feature of budding, the mechanisms used to regulate cell polarity components during mating are largely unknown. The existence of an F-box protein exclusively involved in mating projection development reveals ubiquitination as a means to independently regulate branches of a signal transduction pathway.

P1269**Board Number: B611****Optogenetic control of symmetry breaking in *Saccharomyces cerevisiae*.**K. Witte¹, D. Strickland¹, M. Glotzer¹;¹Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL

Polarization induces the shift from molecular symmetry to asymmetry and is critical to many cellular events including embryogenesis, motility, and cytokinesis. Polarization is inherently dynamic thereby enabling rapid responses to spatial and temporal cues. Optogenetic tools allow functional probing of

cellular polarization and spatial control of signaling molecules. Here, we use the optogenetic tool TULIPs to investigate one of the canonical polarization systems: bud site selection in *Saccharomyces cerevisiae*.

While wild type cells use landmark directed cues to define the polarization axis, cells lacking these spatial cues can spontaneously define an axis in a process known as symmetry breaking. The current model of symmetry breaking suggests that a stochastic accumulation of the active form of the Rho family GTPase, Cdc42, induces a positive feedback loop mediated by a polarity complex containing the Cdc42 GEF, Cdc24, the p21-activated kinase, Cla4, and a scaffold protein, Bem1. Though this model is consistent with a large body of data, it relies on a number of assumptions and it has not been directly tested.

Using TULIPs, we found that local accumulation of either Bem1 or Cdc24 is sufficient to induce recruitment of polarity regulators and effectors, and to promote bud emergence. The ability of Cdc24 to induce polarization depends on its GEF activity. These results are consistent with existing models. However, we find that the polarity proteins do not always function in a potent positive feedback loop. Specifically, membrane recruitment of Cdc24 induces Cdc42 activation early in the cell cycle, but Bem1 does not accumulate until late G1. Although Bem1 recruitment can induce Cdc42 activation in late G1, it is unable to do so during early G1. These results indicate that Cdc42 activation does not invariably induce Bem1-mediated positive feedback. Furthermore, we demonstrate that the switch to Bem1-mediated positive feedback activation requires Cdk1 kinase activity.

Optogenetics allows facile control of competing sites of polarization. We find that Cdc24-generated sites in early G1 can be self sustaining, but that they are readily squelched by a second site in the same cell. Conversely, in late G1, once Bem1 has been recruited, a single site of Cdc24 recruitment cannot outcompete an existing site. Collectively, our study reveals unexpected behaviors of the yeast Cdc42 activation module and demonstrates the utility of optogenetics in dissecting cellular signaling.

P1270

Board Number: B612

The Sequential Logic of Polarity Determination During the Haploid-to-Diploid Transition in *S. cerevisiae*.

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In many organisms, the geometry of encounter of haploid germ cells is arbitrary. In *S. cerevisiae*, the resulting zygotes have been seen to bud asymmetrically in several directions as they produce diploid progeny. What mechanisms account for the choice of direction and do the mechanisms directing polarity change over time? Distinct subgroups of cortical "landmark" proteins guide budding by haploid vs diploid cells, both of which require the Bud1/Rsr1 GTPase to link landmarks to actin. We observe that

as mating pairs of haploid cells form zygotes, bud site specification progresses through three phases. The first phase follows disassembly of components from the zone of cell contact and their reassembly to produce a large medial bud. Bud1 is not required for medial placement of the initial bud. The second phase produces a contiguous bud(s) and depends on axial landmarks. As the titer of the Axl1 landmark diminishes, the third phase ultimately redirects budding toward terminal sites and is promoted by bipolar landmarks. Thus, following the initial random encounter that specifies medial budding, sequential spatial choices are orchestrated by the titer of a single cortical determinant that determines whether successive buds will be contiguous to their predecessors.

P1271

Board Number: B613

Role of conserved NDR kinase Orb6 in translational control and the morphological response to caloric restriction.

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NDR kinases are a highly conserved family of proteins that regulate morphogenesis, cell growth and proliferation, mitosis, and development. Our previous work has demonstrated a role for the fission yeast NDR kinase Orb6 in cell polarization through the spatial control of Cdc42 GTPase activation (Das et al, Curr Biol, 2009). Here we report a genetically separable function for Orb6 kinase in promoting cell viability by preventing translational repression. We found that Orb6 kinase phosphorylates the mRNA-binding protein Sts5, and that *sts5Δ* delays the morphological response to caloric restriction. Specifically, glucose deprivation leads to Sts5-GFP colocalization with Dcp1-mCh, which is a marker of cytoplasmic P-body structures where mRNAs are stored and/or degraded during stresses such as nutrient deprivation or osmotic stress. In addition, chemical inhibition of the analog-sensitive Orb6-as2 mutant kinase also resulted in Sts5-GFP colocalization with P-bodies, mimicking the glucose-deprivation response. Microarray analysis identified several classes of mRNAs that are more abundant in the *sts5Δ* strain, including transcripts involved in cell polarization and bipolar growth activation, cell wall formation, nutrient transport, and meiosis. qPCR analysis confirmed that *sts5Δ* increased levels of selected transcripts and that these levels were decreased upon Orb6-as2 kinase inhibition. We also found that the glucose-sensing PKA pathway synergizes with the Orb6 kinase pathway in the control of P-body formation. These results suggest that Orb6 kinase mediates translational control of specific transcripts by inhibiting the localization of the mRNA-binding protein Sts5 to P-bodies, thus promoting polarized cell growth in cooperation with nutritional response pathways.

P1272**Board Number: B614****Role of the microtubule cytoskeleton in the control of Cdc42 oscillatory dynamics.**M. Rodriguez¹, I. Suarez¹, F. Verde¹;¹University of Miami Miller School of Medicine, Miami, FL

Schizosaccharomyces pombe is a great model system to understand the signaling mechanisms regulating polarized cell growth. Upon the completion of mitosis, *S. pombe* cells grow in a monopolar fashion from their old tips in a process termed Old End Takes Off (OETO). Once the cell reaches a certain length, growth is activated in the second tip through a process called New End Take Off (NETO), resulting in bipolar growth.

Active Cdc42 GTPase, a key regulator of cell polarity, displays oscillatory dynamics that are anti-correlated at the two cell tips in fission yeast. This system globally controls active Cdc42 distribution, modulating cellular dimensions, and controlling the activation of growth at the secondary growing tip (Das, M. et al., *Science*, 2012). Mathematical modeling and experimental analysis suggest Cdc42 dynamic oscillations result from the coordinated function of Cdc42 regulators, including Cdc42 GEF proteins, Gef1 and Scd1, Cdc42 GAP protein, Rga4, and Cdc42-dependent Pak1 kinase, in an interplay of positive and negative feedback mechanisms.

Here, we report that the microtubule cytoskeleton is required for Cdc42 GTPase oscillations. Cells treated with the microtubule depolymerizing agent MBC show asymmetrical distribution of active Cdc42 and delay bipolar growth activation (NETO). Similarly, cells expressing a mutated form of the microtubule tip-associated protein Tea4, which is unable to target the phosphatase Dis1 to the growing tip, displayed asymmetrical Cdc42 activity, a phenotype that is also observed with *gef1Δ* mutants. We find that conserved NDR kinase Orb6 negatively regulates Cdc42 GEF Gef1, an orthologue of mammalian TUBA/DNMBP. A mutated form of Cdc42 GEF Gef1 that cannot be phosphorylated by Orb6 kinase can bypass the microtubule-mediated requirement for NETO. These results provide evidence that the self-organizing microtubule cytoskeleton informs Cdc42 regulation.

P1273**Board Number: B615****Effect of Connexin 43 Loss on Apical Polarity and Differentiation of Normal Breast Epithelium.**R.T. Talhouk¹, D. Bazzoun¹, S.A. Lelievre², H. Adissu²;¹Biology Department, American University of Beirut, Beirut, Lebanon, ²Purdue Univ, West Lafayette, IN

Recent evidence suggests a regulatory role for Connexin (Cx) 43, a gap junction (GJ) protein, in apical polarity establishment that is a key property of epithelial tissues and is disrupted early on during

tumorigenesis. We have previously demonstrated a Cx43 context-dependent tumor-suppressive role mediated partially by a GJ complex assembly that sequesters Cx43-associated proteins β -, α -catenin and ZO-1 proteins at the cell membrane of breast epithelial tumor cell lines and contributes to lumen formation and maintenance in non-neoplastic breast epithelial cells. However, the mechanism that governs Cx43 role in regulating tumor suppression is not fully elucidated. In this study, HMT-3522 S1 non-neoplastic breast epithelial cells were used to decipher the mechanism through which Cx43 contributes to the homeostasis of the normal mammary epithelium. For this purpose, Cx43 was stably silenced in S1 cells using Cx43-specific shRNA along with a nonspecific (NSS) shRNA as control. S1 cells were cultured in three-dimensional (3D) conditions that permit the formation of physiologically relevant epithelial glandular structures (acini). A significant rise in the proliferation rate of S1 cells was noted in response to Cx43 silencing, as reflected by an increase in the size of S1 acini. Enhanced proliferation was accompanied by improper acinar morphogenesis and loss of the ability of S1 cells to form monolayered acini. Moreover; preliminary data from invasion assays revealed that the loss of Cx43 gives the capability for S1 cells to invade, through a layer of diluted Matrigel, to a higher extent than the control and T4-2 cells, the tumorigenic counterparts of S1 cells. These changes, in addition to the disruption of apical polarity in acini and mislocalization of both ZO-1, an apical polarity marker, and β -catenin, a protein involved in the control of epithelial to mesenchymal transition (EMT), indicate a shift in the phenotype into one that enables neoplastic development. Furthermore, Cx43 silencing altered the apico-lateral localization in S1 acini of the protein Scrib, a key regulator of apical polarity and a tumor suppressor recently reported to be involved in the control of EMT in murine lens epithelium and in the regulation of murine mammary gland progenitor activity. We propose that Cx43 tumor-suppressive role may be mediated through an impact on pathways involved in EMT.

P1274

Board Number: B616

Prickle-3 defines the vertebrate PCP axis and controls ciliogenesis during embryonic development.

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Core PCP proteins, including vertebrate homologues of *Drosophila* Van Gogh and Prickle (Pk), maintain planar polarity in a number of vertebrate epithelial tissues and have been implicated in cilia positioning and growth in vertebrate embryos, but the underlying molecular mechanisms remain unclear. Prickle-3 (Pk3), an understudied member of the Pk family, has been shown to associate with the centrosome. Here we demonstrate a striking polarization of Pk3 in *Xenopus* embryonic ectoderm along the anterior-posterior body axis. This polarization of Pk3 in gastrula ectoderm was under the instructive influence of non-canonical Wnt ligands. The C-terminal domain of Pk3 was both necessary and sufficient for the plasma membrane recruitment of Pk3 by Van Gogh-like 2 (Vangl2), but not for Pk3 polarization. In loss-of-function studies Vangl2 homologues have been shown to regulate posterior positioning of cilia in the

frog gastrocoel roof plate (GRP) and the mammalian node, without affecting ciliary growth. By contrast, the knockdown of Pk3 from early *Xenopus* embryos altered both the location and the length of cilia in GRP cells. In cells depleted of Pk3, Vangl2 polarization was disrupted, suggesting that Pk3 functions to maintain Vangl2 distribution and regulate ciliogenesis. Mutagenesis studies attempting to discriminate these two functions of Pk3 will be discussed.

P1275

Board Number: B617

Plasma membrane PI(4,5)P2 levels are critical for actin assembly and polarity during both migration and cell division.

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We monitored signaling and cytoskeletal elements during polarity reversals of migrating *Dictyostelium discoideum* cells using a novel microfluidic device. We found that “backness” and “frontness” in regions of the cell were regulated by plasma membrane (PM) PI(4,5)P2 levels. PI 3’Kinase (PI3K) and Phospholipase C (PLC) activity reduce PI(4,5)P2 levels at the front, while high PI(4,5)P2 levels contribute to “backness”. The tumor suppressor PTEN catalyses the dephosphorylation of the 3’phosphate of PI(3,4,5)P3, producing PI(4,5)P2. PTEN contains a PI(4,5)P2 binding motif and helps maintain high PI(4,5)P2 levels at the back of the cell in a positive feedback loop. Interestingly, the activity of the GTPase Ras was reciprocally regulated with local PI(4,5)P2 levels during polarity reestablishment, suggesting that high PI(4,5)P2 levels inhibit Ras activity, supporting a negative feedback loop. Cells lacking PLC and treated with PI3K inhibitors are still able to reduce PI(4,5)P2 levels and break symmetry when receptors are activated, suggesting that other 4’ or 5’ phosphatases are activated by chemoattractant and contribute to “frontness”, providing yet another level of redundancy.

During cytokinesis, PM PI(4,5)P2 levels rise uniformly as cells round up at metaphase and contribute to the rounding up of the cell. These intermediate levels help reset polarity. PI(4,5)P2 levels subsequently rise in the furrow and are lowered at the poles triggering differential actin assembly, largely through localizing factors specific to the activity of the Rho GTPases, with Arp2/3 mediated filaments forming at the poles. Stimulating metaphase PTEN null cells with chemoattractant gives a transient PI(3,4,5)P3 and F-actin response. Interestingly, lack of PTEN leads to elevation of PI(3,4,5)P3 levels, but they are still regulated in response to chemoattractants at metaphase, suggesting that the threshold for Ras activity is different at this stage. These findings have important implications for cancer. Therapeutic strategies should consider targeting a host of enzymes that regulate PI(4,5)P2 levels, as cancers with low PM PI(4,5)P2 are likely to be highly metastatic.

We have suggested that PI 4’ and 5’ Kinases help terminate uniform chemoattractant-induced responses and contribute to the “backness” of migrating cells. The enzymatic activity that regulates increased PM PI(4,5)P2 levels is likely the inhibition component in the Local Excitation/Global Inhibition model that we

have proposed regulates chemotaxis. The responses seen during a global stimulation and the spatial distribution of signaling molecules when cells are in a chemical gradient may be explained largely by changes in PM PI(4,5)P2 levels, which influence actin assembly and cell morphology.

P1276

Board Number: B618

The MEX-5/6 concentration gradient controls PIE-1 segregation in the cytoplasm of the *C. elegans* zygote.

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Cell polarity is characterized by the asymmetric distribution of factors at the cell cortex and in the cytoplasm. While mechanisms that control the segregation of cortical proteins have been well characterized, less is known about how cytoplasmic proteins become asymmetrically distributed. During the asymmetric division of the one-cell *C. elegans* embryo, a collection of cytoplasmic CCH finger RNA binding proteins are partitioned along the anterior/posterior axis into opposing concentration gradients in response to the conserved polarity regulators, the PAR proteins. MEX-5/6 form a 3-fold anterior-rich concentration gradient while PIE-1, POS-1 and MEX-1 form 5-fold, 3-fold and 2-fold posterior-rich gradients, respectively (Guedes 1997, Mello 1996, Tabara 1999, Schubert, 2000 Ogura, 2003). It was previously demonstrated that the PIE-1 gradient results from its differential diffusion. An increase in PIE-1 diffusion in the anterior cytoplasm causes PIE-1 to be retained preferentially in the posterior where diffusion remains slow (Daniels 2009). The mechanisms that control PIE-1 diffusion are not known.

Using Fluorescence Correlation Spectroscopy analysis fit to anomalous diffusion models, we find that GFP::PIE-1, GFP::POS-1 and GFP::MEX-1 all have significantly slower rates of diffusion in the posterior cytoplasm compared to the anterior cytoplasm. The degree of differential diffusion correlates with the strength of their concentration gradients. For example, GFP::PIE-1 has an apparent diffusion rate of $7.0 \mu\text{m}^2/\text{s}$; $a = 0.64$ in the anterior and $0.4 \mu\text{m}^2/\text{s}$; $a = 0.35$ in the posterior whereas MEX-1 has an apparent diffusion rate of $1.5 \mu\text{m}^2/\text{s}$; $a = 0.51$ in the anterior and $0.5 \mu\text{m}^2/\text{s}$; $a = 0.42$ in the posterior (a is the anomaly coefficient). MEX-5/6 are epistatic to PAR-1 and PAR-3 with respect to the control of PIE-1 mobility, indicating that MEX-5/6 likely act downstream of PAR proteins. We progressively depleted MEX-5/6 levels in *par-1(it51)* mutant embryos by partial RNAi and find that PIE-1 diffusivity correlates with the concentration of MEX-5/6. These data support a model in which the MEX-5/6 concentration gradient controls the establishment of a PIE-1 diffusion gradient, which in turn, leads to the segregation of PIE-1.

Neuronal Cytoskeleton 1

P1277

Board Number: B620

Examining the Roles of Myosin II, Dynein, and Drebrin in Nucleokinesis of Migrating Neurons.

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Migration of neurons from their germinal zone to final location is an essential process in the developing brain. Following polarization of a developing neuron, nucleokinesis occurs in a two-stroke process. The nucleokinesis cycle is the saltatory motion of centrosome and cytoplasm transport into the leading process dilation of a neuron followed by somal translocation. A current debate in the field revolves around the roles of the actin and microtubule networks in the two-stroke nucleokinesis cycle and how they generate force within the cell.

We examined cytoskeleton elements and motors involved in neuronal migration through *in vitro* time-lapse imaging using the mouse cerebellar granule neuron system, conditionally perturbing motor function using small molecule inhibitors. We examined potential modes of actin/microtubule crosstalk through functional studies of drebrin, which interacts with both microtubule plus tips and actin. The leading process dilation of granule neurons is enriched with myosin II motors during the two-stroke nucleokinesis cycle and live imaging of venus tagged dynein motors showed that dynein flows from the soma toward the leading process dilation. We also show the leading process dilation is enriched with drebrin immediately preceding somal translocation.

Pharmacological inhibition of myosin II motor ATP-activity using blebbistatin retards movement of the centrosome. Using HPI-4 (Ciliobrevin A) to similarly inhibit dynein, organelle dynamics were tracked in migrating neurons using the centrosome, cilia, golgi, and nucleus as metrics to compare the effects of inhibiting myosin II to dynein. Single inhibition of myosin II or dynein motors slowed all organelles tested, however residual movement remained. While single inhibition of myosin and dynein phenocopied each other, dual inhibition of both myosin II and dynein completely halted organelle positioning and migration.

Functional inhibition of drebrin through dominant-negative constructs, shRNA, and pharmacologically with BTP2 all resulted in similar disruption of directed migration of granule neurons *in vitro*. Population analysis of drebrin inhibited cells show a lower occurrence of migration, and cells that migrated moved inefficiently. Leading process cytoskeletal dynamics of migrating neurons was examined in drebrin inhibited cells.

Overall these results indicate that both myosin II and dynein motor systems dually contribute to neuronal migration in the leading process of migrating granule neurons, potentially through drebrin linking actin and microtubule networks.

P1278

Board Number: B621

RhoGTPase Regulatory Signatures Define Distinct Stages of Synaptic Development.

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Small RhoGTPases organize the actomyosin cytoskeleton to drive changes in post-synaptic spine shape that support learning and memory. RhoGTPase signaling pathways are major targets of synaptic disorders, such as Autism. During normal synaptic development, the small RhoGTPase, Rac, promotes the formation of filopodia-like spine precursors, which subsequently mature through RhoA-dependent myosin II activation into polarized mushroom-shape spines. Further excitatory stimulation associated with long-term potentiation leads to Rac-driven spine head expansion. We sought to identify novel regulators of synaptic RhoGTPase activity, and to determine whether different upstream RhoGTPase regulatory proteins, including GEF activators, GAP inactivators, and inhibitory GDIs, regulate specific stages of synaptic development. We performed an in-silico screen of genes identified from published synaptic proteomes and the SFARI database for association of these genes with Autism copy number variants (CNVs) to identify known and putative RhoGTPase regulatory proteins. This approach identified several candidate actin regulatory proteins, the majority of which localized to known Autism CNVs. We used shRNA to down-regulate expression of specific RhoGTPase regulatory proteins in rat primary hippocampal neurons during either early spine formation or subsequent maturation. Our data demonstrate that unique RhoGTPase regulators mediate distinct stages of synaptic development. Specifically, the Rac-GEF beta-Pix, ArhGAP23 and Frabin, affected early spine precursor formation, but not subsequent maturation. However, GDI-mediated attenuation of RhoGTPase activity stabilized mature spines; knockdown of either GDI-alpha or gamma resulted in significantly longer spines. The novel RhoGAP, ArhGAP39, regulated spine length and density specifically during spine maturation. Notably, RNA expression of these GDIs and ArhGAP39 increased significantly during neuronal development. We further characterized the function of the putative RhoGAP, ArhGAP39, in migratory CHO.K1 fibroblasts, which also express ArhGAP39. GFP-ArhGAP39 resulted in robust stress fiber formation and focal adhesion maturation, consistent with GAP activity towards Rac, resulting in a corresponding increase in RhoA-mediated myosin activation. Alternately, knockdown of ArhGAP39 resulted in round cells with an extensive lamellipodia associated with increased small nascent adhesions, indicative of increased Rac activity, which we confirmed with a Rac FRET biosensor. Our study demonstrates that specific combinations of RhoGTPase regulatory proteins temporally balance Rac and

RhoA activity to regulate post-synaptic spine development, and further shows that ArhGAP39 functions as a novel Rac GAP in post-synaptic spine maturation.

P1279

Board Number: B622

Regulation of actin dynamics by RhoA-LIMK-cofilin during axon retraction by rod photoreceptors after retinal injury.

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In response to injury, rod photoreceptors display synaptic plasticity including axon retraction and synaptic disjunction. Our previous studies demonstrated that the mechanism of retraction involves activation of RhoA and its downstream effector Rho kinase (ROCK). Here, we pursued further downstream events in order to develop possible therapies.

First, we measured axon retraction of isolated salamander rod cells over time with or without inhibition of LIM kinase, a downstream effector of ROCK. Our results showed that a LIMK inhibitor (LIMKi) can block retraction: 38% decrease of initial rod axon length in controls after 7 hours, compared with only a 25% decrease using 10uM LIMKi. We next studied the activity of cofilin, an effector of LIMK which regulates actin filament turnover. We created detachments of pig retina, incubated the retinal explants for 24 hours and examined both total and phosphorylated cofilin with Western Blots. P-cofilin increased over 24 hours whereas total cofilin remained relatively stable; treatment with LIMKi or the ROCK inhibitor Y27632 prevented such increases.

Since activity of cofilin leads to an increased turnover rate of actin filaments, we were curious whether or not actin depolymerization contributes to axon retraction in rod photoreceptors. We examined actin filaments by phalloidin staining and the barbed end assay. Isolated salamander rod cells were stained with phalloidin-Alex488 at 15 minutes, 2, 4, and 7 hours after retinal dissociation. Quantification of fluorescence signal suggested that after an initial increase during the first 2 hours (about 2 fold), actin filaments then depolymerize indicated by a 30% decrease in fluorescence intensity by 7 hours. The barbed end assay measures freshly severed actin filaments by labeling them with a biotin-actin monomer, which demonstrates activity of cofilin/p-cofilin. Severing of actin filaments was highest at 15 minutes and then decreased by 80% during the remaining 7 hours, likely attributable to fewer available actin filaments over time. Pretreatment of retina with either ROCK or LIMK inhibitor significantly reduced barbed end labeling 15 minutes after injury, by 70% and 90% respectively.

Immunocytochemistry also showed co-localization of p-LIMK, the active form of LIMK, with barbed end actin filaments in the axonal region of rod photoreceptors during retraction.

To summarize, our data showed that the RhoA-ROCK-LIMK-cofilin pathway is involved in axon retraction of rod photoreceptors after retinal detachment and works in part by up-regulating actin

depolymerization. Inhibition of this pathway can prevent axon retraction and stabilize synaptic structure presumably by stabilizing the actin filament cytoskeleton.

P1280

Board Number: B623

Rear accumulation of actin underlies nucleus-centrosome inversion type of nucleokinesis in migrating neocortical neurons.

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Neuronal migration is an integrative process involving leading process formation, nucleokinesis, and rear retraction, all of which depend on the function of cytoskeletons. Cytoplasmic microtubules (MTs) and dynein have been implicated in force generation for nucleokinesis. However, several lines of evidence suggest that the actomyosin system likely functions in co-operatively. To assess the contribution of MT and actin cytoskeletons in nucleokinesis, we employed *in situ* live imaging of migrating neocortical neurons in slice culture (Sakakibara et al. (2014) *Cereb. Cortex* 24:1301-1310). Lifeact-EGFP (F-actin), PACT-mKO1 (centrosome), and Histone-H2B-iRFP670 (nucleus) were used for simultaneous labeling and imaging by triple color fluorescence channels. During the locomotion of neurons, two types of nucleokinesis were observed (reviewed in Sakakibara et al. (2013) *Open Biol.* 3:130061). The first one is the nucleus-centrosome (N-C) coupling type of nucleokinesis. Centrosome proceeded into the leading process prior to the N-C coupling type of nucleokinesis; this move has been implicated in the dynein-dependent forward pulling of nucleus by MTs. Rear accumulation of F-actin was not necessarily observed during the N-C coupling type of nucleokinesis. The second type of nucleokinesis is the N-C inversion during which translocating nucleus overtake the centrosome. Rear accumulation of F-actin was evidently observed when the N-C inversion occurred. These data suggest that the actomyosin system underlies the N-C inversion type of nucleokinesis during which the MT-dynein system could not pull the nucleus.

P1281

Board Number: B624

A Dynamic Formin-dependent Axonal F-actin Network.

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The cytoskeletal protein actin is involved in numerous aspects of neuronal physiology. Although actin organization and dynamics at growth-cones is well studied, much less is known about axonal actin. Using probes that specifically bind filamentous actin (F-actin), we found local zones of actin assembly/disassembly ('hotspots') distributed along axons. These hotspots are a nidus for vigorous

Formin-dependent actin assembly (“actin trails”) that appears to occur on the surface of stationary axonal vesicles. Intriguingly, the overall kinetics generate an anterogradely-biased flow of the F-actin population into axons and en-passant synapses, at rates consistent with slow axonal transport. Disrupting the Formin-mediated dynamics has little effect on microtubule-based transport, but interferes with axonal elongation and presynaptic function. Collectively, our studies reveal previously-unknown mechanistic details of an axonal actin-network with implications for neuronal physiology. We propose a model where an overall dynamism maintains axonal actin in an organized yet flexible state – allowing swift response to intrinsic and extrinsic cues.

P1282

Board Number: B625

Formin -2 in Neuronal Development.

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The establishment of proper neuronal connections during early embryonic development is tightly regulated by instructive cues that guide the exploring neuronal growth cone to find its synaptic targets. The growth cone steering is achieved by cue-dependent remodelling of the underlying cytoskeleton that regulates polarity, protrusion and generation of coordinated traction forces. We have screened the developing chick embryo for various actin nucleators and have identified Formin 2 (Fmn-2) expression to coincide with the window of spinal commissural neurite outgrowth and floor plate crossing. In vivo knockdown of Fmn2 results in defective midline crossing by the commissural interneurons. Using cultured primary neurons, we show that Fmn2 is involved in regulating growth cone motility. Studies in fibroblasts reveal that Fmn2 is involved in stabilizing cell-substrate interactions. Taken together our study identifies Fmn2 as central regulator of growth cone dynamics central to axonal outgrowth and pathfinding .

P1283

Board Number: B626

Molecular architecture of the Axon Initial Segment revealed by super-resolution microscopy.

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The Axon Initial Segment (AIS), located within the first thirty microns of the axon, has two essential functions in neuronal physiology. First, it generates the axon potential through the concerted action of voltage-gated ion channels. Second, it maintains neuronal polarity by separating the somatodendritic and axonal compartments. This separation is ensured by a diffusion barrier at the AIS membrane, and a proposed intracellular transport filter that regulates cargo access to the axon. The molecular

composition of the AIS has been characterized in the recent years, and depends on the assembly of the ankyrin G (ankG) / β IV-spectrin scaffold. However, we don't know how AIS components are arranged to form the surface barrier and the transport filter. This motivated us to assess the molecular architecture of the AIS by elucidating the nano-organization of the scaffold proteins ankG and β IV-spectrin.

We used STochastic Optical Reconstruction Microscopy (STORM), which provide a ~ 10 times enhancement in spatial resolution over diffraction-limited microscopy. STORM recently uncovered a striking periodic submembrane structure for spectrin and actin in the axon. Using 2-color STORM on cultured hippocampal neurons, we detected rings of actin spaced every 185 nm along the AIS, connected by a periodic submembrane sheath of longitudinally-oriented β IV-spectrin proteins. Thanks to antibodies directed against several domains of the large ankG isoforms, we then mapped ankG positioning in the AIS. The spectrin-binding domain of ankG, located on its amino-terminal side, is organized periodically along the β IV-spectrin sheath. Strikingly, the opposite carboxy-terminal domain of ankG showed a less organized distribution, hinting at an extended conformation of ankG. Interestingly, 2-color 3D-STORM showed that the carboxy-terminus of ankG localizes further inside the AIS axoplasm than its spectrin-binding domain. Quantification of 3D-STORM images allowed us to determine a radial extension of 40 nm between these two ankG domains. Finally, the two features of the AIS nano-architecture, longitudinal β IV-spectrin and radial ankG, are robust to acute perturbation of the actin and microtubule cytoskeleton. Resolving the molecular architecture of the AIS allow us to foster new hypotheses on how the AIS scaffold can regulate protein transport and maintain polarity in neurons.

P1284

Board Number: B627

Nuts and bolts of the axon initial segment cytoskeletal architecture: Roles of β IV-spectrin.

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The axon initial segment (AIS) is a specialized compartment in polarized neurons essential for regulating action potential initiation and axon dendrite polarity. AIS function depends on the formation and maintenance of a specialized cytoskeleton at the plasma membrane, but the function of individual cytoskeletal components in the AIS remains unclear. Using platinum replica electron microscopy (PREM), we show that the AIS is highly organized into a multi-protein network containing plasma membrane integral proteins (e.g., ion channels and cell adhesion molecules), as well as core cytoskeletal components (e.g., ankyrinG, β IV-spectrin, and actin filaments) that have been hypothesized to serve as a molecular platform on which all other AIS proteins are organized and maintained. While it is clear that ankyrinG function as a master organizer responsible for the recruitment and stability of all other AIS proteins, the role of β IV-spectrin is unclear. Using immunoPREM combined with shRNA-based depletion methods, we investigate the role of β IV-spectrin as a key component of the core AIS cytoskeletal backbone and its role in the overall organization, assembly and maintenance of the AIS multi-protein

network. Consistent with recent studies, we show that β IV-spectrin is essential for long-term AIS maintenance and stability by promoting the retention of AIS-enriched proteins. Intermediate stages of AIS cytoskeleton disassembly following β IV-spectrin depletion reveal new features of the AIS cytoskeletal architecture.

P1285

Board Number: B628

Spectrin β III functions at the neck and base of dendritic spines to support their formation in hippocampal neurons.

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The neurodegenerative disorder spinocerebellar ataxia type 5 (SCA5) is linked to mutations in the SPTBN2 gene encoding a neuron-specific spectrin β III. At the cellular level, spectrin β III deficiency impairs formation of dendritic spines and reduces accumulation of some postsynaptic components, but underlying mechanisms remain unclear. The shape of the spine head is maintained by a dense branched actin network, whereas a cytoskeletal mechanism supporting for the constricted shape of the spine neck remains unclear, because of the absence of adequate actin cytoskeleton in the spine neck [Korobova & Svitkina, 2010, Mol. Biol. Cell, 21, 165-176]. Here, we show that spectrin β III in cultured rat hippocampal neurons is enriched at the base and neck of dendritic spines, where it comprises a part of the detergent-resistant cytoskeleton and exists in the form of thin fibrils with characteristic dimensions of spectrin tetramers. Silencing of spectrin β III markedly decreases formation of dendritic spines and postsynaptic specializations without significant effects on formation of presynaptic boutons. Accordingly, spectrin β III knockdown neurons exhibit increased occurrence of unopposed presynaptic boutons and boutons apposing weak postsynaptic structures in a dendrite shaft. We propose that spectrin β III functions as a part the membrane cytoskeleton to maintain the dendritic spine neck, which forms a diffusion barrier retaining postsynaptic molecules at the synapse. In the absence of the neck, increased diffusion of postsynaptic components leads to formation of weak synapses or their absence. Thus, our data significantly advance understanding of the molecular mechanisms of SCA5 and reveal parallels between spectrin functions in neurons and erythrocytes.

P1286**Board Number: B629****Regulation of neurofilament transport by a dynamic cycle of polymer severing and annealing.**A. Uchida¹, A. Brown¹;¹Department of Neuroscience, The Ohio State University, Columbus, OH

Neurofilaments are transported and distributed along axons by the mechanisms of axonal transport, moving anterogradely and retrogradely in rapid infrequent bursts of movement interrupted by prolonged pauses. We have discovered that axonal neurofilaments lengthen by joining end-to-end, called end-to-end annealing, and that they can also be shortened by a severing mechanism. To explore the significance of these novel phenomena for neurofilament transport, we developed a long-term multi-field tracking method that uses a motorized stage to follow single GFP-tagged neurofilaments along axons by time-lapse fluorescence microscopy for hundreds of μm across multiple fields of view. The tracked filaments ranged from 1.3 to 56.0 μm in length. Short filaments (20 μm in length; average=29.5 μm , n=51) exhibited more frequent severing (0.15 annealing events/filament/hour; 1.5 severing events/filament/hour). Filaments of all lengths were capable of rapid movement, but short filaments moved more persistently, pausing less and rarely reversing direction, whereas long filaments moved less persistently, pausing for long periods, and often reversing direction. Thus the kinetics of neurofilament transport are influenced by the length of these polymers. Since N-terminal phosphorylation is known to result in fragmentation and disassembly of intermediate filaments, we used site-directed mutagenesis to investigate whether N-terminal phosphorylation might regulate neurofilament length. GFP-fusions of NFL in which serines 2, 55, 57 and 66 in the N-terminal head domain were all mutated to either alanine (S4A-NFL) or aspartate (S4D-NFL) both assembled into filaments, contrary to previous reports, but filaments containing S4A-NFL were longer (average=13.5 μm , n=87) and moved less frequently (average=4.5/hour, n=23 axons) than wild type, whereas filaments containing S4D-NFL were shorter (average=5.1 μm , n=146) and moved more frequently (average=9.6/hour, n= 28). We propose that the length and transport of neurofilaments in axons is regulated by a dynamic cycle of polymer severing and annealing. Severing liberates short filaments that move more readily, whereas end-to-end annealing of these short filaments sequesters them in the form of long polymers that move less readily. We speculate that site-directed N-terminal phosphorylation of neurofilaments may be a mechanism for intermediate filament severing. We are currently testing this hypothesis.

P1287**Board Number: B630****A giant and KASH-less isoform of Nesprin1 underlies the molecular etiology of autosomal recessive cerebellar ataxia Type 1 (ARCA1).**D.M. Hodzic¹, D.S. Razafsky¹;¹Ophthalmology and Visual Sciences, Washington University School of Medicine, Saint Louis, MO

Linkers of the Nucleoskeleton to the Cytoskeleton (LINC complexes) designate evolutionary-conserved macromolecular complexes that span the nuclear envelope (NE) and physically connect the nuclear interior to cytoskeletal networks and molecular motors. LINC complexes assemble within the perinuclear space through direct interactions between the SUN domain of inner nuclear membrane Sun proteins (Sun 1 and Sun 2) and the KASH domain of outer nuclear membrane Nesprins (Nesprin 1, 2, 3 and 4). Nesprin1giant is encoded by a >27kb transcript which contains several internal transcription initiation and termination sites resulting in the expression of numerous smaller Nesprin1 variants. Patient's harboring mutations along the entire length of nesprin1 develop autosomal recessive cerebellar ataxia type 1 (ARCA1), a late onset disease characterized by diffuse cerebellar atrophy and progressive loss of coordination in movements and speech. Despite the identification of such mutations of nesprin1, the molecular etiology of ARCA1 remains unknown. Our characterization of LINC complexes in cerebellum indicates that Nesprin1 is present as nuclear rims exclusively in Purkinje cells. However, we found that in vivo disruption of LINC complexes in Purkinje cells has no effect on cerebellar development and mice display no symptoms of cerebellar ataxia. To date, Nesprin1 has only been studied in the context of its association with the NE; however, we recently discovered that the alternative splicing of Nesprin1 penultimate exon leads to the synthesis of KASH-less isoforms, an event that is strikingly predominant in CNS tissues. In agreement with our in situ hybridization results, which indicate that Nesprin1 transcripts are extremely abundant in the granule cell layer, we observed a strong Nesprin1 immunoreactivity in the shape of large speckles within the granule cell layer. These speckles correspond to cerebellar glomeruli, which are complex "synaptic hubs" composed of axonal and dendritic terminals of mossy fibers and granule cells, respectively as well as axonal and dendritic terminals of cerebellar Golgi cells. We therefore propose that ARCA1 mutations affect the essential function that a giant KASH-less isoform of Nesprin1 giant plays at cerebellar glomeruli. We are currently evaluating several mouse models that could provide additional details about the non-canonical functions of Nesprin1. These models may prove helpful to better characterize ARCA1 and to evaluate new therapeutics.

P1288**Board Number: B631****Shaping neurons by twist-tension coupling in chiral cytoskeleton networks.**M. Krieg¹, J.G. Cueva², M.B. Goodman²;¹Molecular and Cellular Physiology, Stanford University, Stanford, CA, ²Molecular Cellular Physiology, Stanford University, Stanford, CA

Many somatosensory neurons have evolved specialized molecular sensors that convert mechanical stress into behavioral responses. The genetics, development and physiology of the touch receptor neurons (TRNs) in *Caenorhabditis elegans* nematodes are especially well characterized and this animal has the particular advantage that the TRNs can be studied both in living animals and dissociated in culture. Moreover, *C. elegans* is a unique model organism to study the mechanics of neurons due to the low complexity of its nervous system, the known wiring diagram and stereotypic behaviors, thus permitting a systems perspective on cell function.

Like in other animals, the shape of neurons in *C. elegans* is intimately coupled to their function. Some neurons are highly branched and curved, while others are extremely straight. We have previously shown that a functional, pre-stressed spectrin network is critical for mechanosensation and neuron stability under body-evoked forces. How the constituent molecules of these different neurons establish a functional organization and how tiny molecules can determine cell shape that exceeds their size by orders of magnitude is barely understood. We first compared different neurons, and classified their shapes. TRNs were by far the straightest neurons in living animals, and its shape critically depends on functional TRN-specific microtubule bundles and a pre-stressed spectrin network. Specifically, TRNs undergo deformations reminiscent of a helical buckling instability similar to twisted rods under compression. In the limit, spectrin network deficiency leads to shorter microtubule bundles. These results, together with super-resolution data on spectrin cytoskeleton and mechanical modeling of the neuron under compression, suggest that a chiral cytoskeletal network and microtubule bundle mutually stabilize each other to produce an extremely straight cell shape, critical for mechanosensation.

P1289**Board Number: B632****Kinesin-powered microtubule sliding drives axonal regeneration in cultured *Drosophila* neurons.**W. Lu¹, M. Lakonishok¹, V.I. Gelfand¹;¹Department of Cell and Molecular Biology, Northwestern University, Feinberg School of Medicine, Chicago, IL

Previously we demonstrated that microtubule sliding by conventional kinesin (kinesin-1) is required for initial neurite outgrowth in cultured *Drosophila* embryonic neurons, and sliding is developmentally downregulated when neurite outgrowth is completed. Here we report that axotomy of *Drosophila*

neurons in culture triggers regeneration and axonal regrowth. Regenerating neurons contain sliding microtubules; this sliding, like sliding during initial neurite outgrowth, is driven by kinesin-1 and is crucial for axonal regeneration. The injury induces a fast spike of calcium and generation of microtubule arrays with mixed polarity. Both of these events are required for reactivation of microtubule sliding and regeneration. Furthermore, we reveal that the c-Jun N-terminal kinase (JNK) pathway promotes regeneration by enhancing microtubule sliding in mature neurons. Supported by NIGMS 52111.

P1290

Board Number: B633

Acceleration of Growth Cone Advance by Resveratrol Parallels Changes in Microtubule (MT) Acetylation and MT Tip Distribution.

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The process of growth cone advance requires a functional transition between dynamic, deacetylated MTs in the peripheral domain and stable, acetylated MTs (AcMT) in the axon. Resveratrol, a plant-derived polyphenol, activates Sirtuin 2: a NAD⁺ dependent tubulin deacetylase. Previously, we have shown that resveratrol enhances growth cone advance while AKG2, a Sirtuin inhibitor, slows growth cone advance. To determine if resveratrol decreases the amount of AcMTs in neurons, we used immunofluorescent analysis to quantitate the amount and distribution of (AcMTs) relative to all MTs in cultured E18 rat neurons and *Helisoma* neurons. While resveratrol treatment reduced the population of AcMTs in both types of neurons, the changes occurred in different regions of neurons. In E18 rat neurons, we detected a decrease in relative amount of acetylated tubulin in growth cones, while in *Helisoma* neurons, we observed a decrease in acetylated tubulin in axons. To understand how a decrease in AcMTs in the axon modifies growth cone advance, we examined the effect of resveratrol treatment on growth cone MTs in accelerating *Helisoma* growth cones. Our results revealed that the percentage of visualized microtubule tips at the leading edge of the growth cone significantly increased in response to resveratrol. Furthermore, threshold analysis of fluorescent deconvoluted images showed an increase in the percent area of total tubulin staining in the growth cone in response to resveratrol. These results suggest that decreasing AcMT levels in the axon accelerates growth cone advance by promoting advancement of dynamic MTs into the leading edge of growth cones. The inverse relationship between axonal AcMTs and dynamic growth cone MTs may reflect a role for AcMTs in regulating the distribution of MT tip proteins.

P1291**Board Number: B634****Microtubule transport in axons: theory and experiment.**E.M. Craig¹, A. Rao², K.A. Myers³, P.W. Baas²;¹Physics, Central Washington University, Ellensburg, WA, ²Department of Neurobiology and Anatomy, Drexel University College of Medicine, Philadelphia, PA, ³Department of Biological Sciences, Misher College of Arts and Sciences, University of the Sciences, Philadelphia, PA

Microtubules (MTs) align in the axons of nerve cells to create a long parallel bundle of polar filaments extending from the cell body to the growth cone. While the longer filaments in the bundle are stationary, a number of short MTs (1-10 μm) within the bundle undergo rapid bi-directional movements that can be paused by proteins that impose a brake on the movements. The coordinated movement of short MTs along the axon is essential for establishment and maintenance of the MT bundle, and we posit for organizing MTs within the bundle. We used a combination of experimental and computational approaches to investigate how the transport of short MTs within the bundle may contribute to the preservation of its polarity pattern as well as its expansion as axons grow. Here, we present a computational modeling framework developed to investigate how mechanical interactions between molecular motor proteins and MTs produce the patterns of MT transport that have been measured experimentally. We modeled molecular motor-driven movement of individual MTs using stochastic simulations based on force-dependent binding and unbinding rates for subpopulations of molecular motor proteins that are known to interact with and regulate MT transport. We tested the predictions of the model by comparing simulated MT transport events with live-cell imaging of MT transport in developing axons.

Membrane Trafficking at the Synapse**P1292****Board Number: B635****SH3 domain-mediated interactions and F-BAR domain higher-order assembly regulate Nervous Wreck membrane remodeling.**C.F. Kelley¹, E. Messelaar¹, T.L. Eskin^{1,2}, A.A. Rodal^{1,2};¹Molecular and Cell Biology, Brandeis University, Waltham, MA, ²Brandeis University, Waltham, MA

Membrane trafficking requires the coordination of hundreds of interacting proteins, which act in concert to drive cargo sorting, membrane tubulation, fission, and fusion. While well characterized individually, it is not understood how their activities are regulated and targeted in vivo to fulfill diverse roles within cells. Nervous Wreck (NWK) encodes an F-BAR/SH3 protein that regulates the traffic and signaling output of synaptic growth receptors at the *Drosophila* neuromuscular junction (NMJ) through its interactions with the membrane, actin nucleation machinery, and other endocytic proteins including

dynamain and Dap160/Intersectin. Here, we find that the membrane binding and remodeling activities of the Nwk F-BAR domain are autoinhibited through direct interaction with its two C-terminal SH3 domains, and that this autoinhibition is important for Nwk localization and function in vivo. Unlike the isolated F-BAR domain, autoinhibited Nwk strictly requires self-assembly in order to stably interact with and remodel membranes. Previous models for autoregulation of BAR family proteins proposed that SH3 domains directly compete for membrane binding, controlling exchange of the protein between the cytosol and the membrane. We found that the Nwk auto-regulatory mechanism specifically limits promiscuous membrane binding and promotes higher order assembly. Further, we find that the endocytic scaffold Dap160, which regulates Nwk localization at the NMJ, relieves autoinhibition and increases both the rate and frequency with which Nwk associates with the membrane in vitro. These results suggest a complex regulatory mechanism, dependent on separable intra- and inter-molecular interactions, by which membrane remodeling is tightly regulated, and lend insight into the mechanisms employed by the cell to specifically target membrane remodeling.

P1293

Board Number: B636

An impaired expression of ascorbic acid transporter (SVCT2) at cellular surface would contribute to oxidative stress in Huntington's disease.

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Introduction. Huntington's disease (HD) is a neurodegenerative disorder characterised by progressive abnormalities in cognitive and motor control. HD is caused by an expansion in the first exon of the huntingtin gene (Htt). Htt participates as an integrator of vesicular and protein traffic. HD is characterised by an increase in oxidative stress. Ascorbic acid is a powerful antioxidant that is highly concentrated in the brain. Synaptic activity triggers the release of ascorbic acid from glial intracellular reservoirs. Ascorbic acid is transported into neurons via the Sodium-dependent Vitamin C Transporter-2 (SVCT2). In this study, we have examined SVCT2 expression at plasma membrane in cellular models of HD. **Materials and Results** Using immortalized striatal neurons derived from knock-in mice expressing mutant huntingtin (STHdhQ cells) we studied ascorbic acid transport and SVCT2 expression at plasma membrane. When extracellular ascorbic acid concentration increases (as occurs during synaptic activity) SVCT2 is translocated to the plasma membrane ensuring optimal ascorbic acid uptake for neurons. However, in HD cells, SVCT2 fails to reach the plasma membrane. Analyses using endocytosis and exocytosis inhibitors showed that SVCT2 cycling to and from the plasma membrane is impaired in HD cells. Co-immunoprecipitation and colocalization assays confirmed the existence of HAP1-SVCT2 interaction which is missing in HD cells. On the other hand, HD cells showed an increased SVCT2-HAP40 colocalization. Live cell imaging analyses revealed an impairment in ascorbic acid-induced calcium signaling. **Discussion.** Our results suggest that SVCT2 is being recycled constitutively. The loss of Htt

function disrupts SVCT2 translocation to the plasma membrane. Also, HD cells show an impairment in the signaling responsible to induce SVCT2 translocation to cellular surface. Ascorbic acid uptake is essential to maintain redox balance in the brain. Therefore, an early impairment of ascorbic acid uptake in HD neurons could promote neuronal death. Fondecyt 1110571, DID-UACH.

P1294

Board Number: B637

Regulation of synaptic vesicles by the mitochondria-caspase-autophagy pathway.

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Macroautophagy ('autophagy' hereinafter) is a process by which organelles and aggregated proteins are delivered to lysosomes for degradation. Autophagy for organelle degradation comes in various forms, such as mitophagy for mitochondria, ribophagy for ribosomes, pexophagy for peroxisomes, and reticulophagy for endoplasmic reticulum. In multicellular organisms, however, where the number of cells and variety of organelles are greatly increased, except for mitophagy, the physiological significance of autophagy for organelles, especially those specific to multicellular organisms (e.g. synaptic vesicles), remains elusive.

The number of synaptic vesicles at a synapse governs synaptic strength, which is dynamically regulated during development and by experience. A stable, yet flexible, pool of synaptic vesicles is therefore critical to ensure the reliability and adaptability of neural circuits. However, it remains largely unclear how the number of synaptic vesicles is maintained and regulated. Our study finds that autophagy controls synaptic vesicle number. In Atg protein knockout mice where autophagy is reduced, synaptic vesicle pools are enlarged. Conversely, in mice with enhanced autophagy, synaptic vesicle pools are smaller than normal. Moreover, we find that for controlling synaptic vesicles, autophagy is regulated by the BAD-BAX-caspase pathway. We also show that the autophagic trafficking of synaptic vesicles takes place in early and late endosomes. These findings elucidate a new, autophagy-mediated mechanism of synaptic vesicle turnover, and a new, non-apoptotic, presynaptic function for the BAD-BAX-caspase-3 cascade. They also demonstrate that caspase-regulated autophagic trafficking of synaptic vesicles is essential for both the homeostasis and activity-dependent regulation of synaptic vesicle pools.

P1295**Board Number: B638****PGRMC1 expression correlates with the sigma-2 fluorescent probe (SW120) staining in rat hippocampus cells.**C. Zeng¹, N. Garg¹, R.H. Mach¹;¹Radiology, University of Pennsylvania, Philadelphia, PA

Progesterone receptor component 1 (PGRMC1) is identified as the potential sigma-2 receptor binding site. PGRMC1/sigma-2 receptor is elevated in various types of human tumors and is identified as a biomarker for imaging solid tumors using the functional positron emission tomography (PET) technology. However, the function of PGRMC1/sigma-2 receptor in the central nervous system (CNS) is largely unknown. In the current study, we examined the PGRMC1 expression by immunohistochemistry and sigma-2 fluorescent probe (SW120) staining in primary cultures of neurons, astrocytes, oligodendrocytes and microglia cells of E18 rat hippocampus. Our data show that the expression of PGRMC1 and SW120 staining are prominent in neurons, moderate in oligodendrocytes and microglia cells, and very low in astrocytes. These results indicate that PGRMC1 expression levels correlate with sigma-2 receptor densities in four different rat brain cell types, supporting our previous finding that PGRMC1 is associated with the sigma-2 receptor binding site. The results imply that the sigma-2 receptor PET radiotracer can potentially be used to noninvasively image neuron/synapse densities in human CNS.

P1296**Board Number: B639****SYNAPTIC STABILITY AND GLIAL REACTIVITY DURING SENESCENCE IN MDX MICE.**G.F. Simoes¹, L.P. Cartarozzi², A.B. Spejo², A.L. Oliveira^{2,3};¹Faculty of Medical Sciences of the Santa Casa of São Paulo, São Paulo, Brazil, ²Structural and Functional Department, University of Campinas, Campinas, Brazil, ³Univ Campinas, Campinas, Brazil

Duchenne muscular dystrophy (DMD) causes degeneration of skeletal muscle fibers which can result in retrograde changes that affect the central nervous system. In this sense, spinal motoneurons show the typical signs of chromatolysis and loss of synapses in the early stages of disease. Further degeneration may occur with the aging process, although it has been poorly investigated. In this sense, the present work studied motor function and spinal cord microenvironment during senescence in MDX mice. C57BL/10 mice were used as the control strain. For this, we used fifteen mice from each strain, which were evaluated at six, twelve, and eighteen months old. The motor function was monitored using the walking track test (Catwalk system). The animals were sacrificed and the spinal cords processed for immunohistochemistry (GFAP and synaptophysin antisera). The results showed that MDX mice present a significant (107%) motor deficit as compared to control animals at six months old, that further

decreased at twelve months (80%) and eighteen months (39%). The Max Contact Max Intensity decreased 13%, 19% and 21% in MDX mice at six, twelve, and eighteen months, respectively. GFAP immunolabeling increased in both strains during senescence, being more evident in all groups of MDX mice. Synaptophysin immunolabeling decreased in MDX mice in all groups studied. Overall, the results herein indicate that the constant cycles of muscle degeneration/regeneration in MDX mice cause retrograde structural changes in the spinal cord microenvironment, directly affecting the homeostasis of the CNS during the course of Duchenne muscular dystrophy.

P1297

Board Number: B640

Ubiquitin-mediated regulation of diverse small-molecule receptors in *C. elegans*.

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Ubiquitin-mediated protein regulation is vital for general cellular maintenance, and, in particular, for neural function. In the model organism, *C. elegans*, we have shown that the deubiquitinating enzyme, USP-46, works in concert with two WD-40 domain proteins to regulate the abundance and trafficking of the glutamate receptor, GLR-1. Specifically, USP-46 is activated by WDR-20 and WDR-48 to deubiquitinate and stabilize GLR-1. Modulation of USP-46's activity via these WDR proteins determines the extent of ubiquitination on GLR-1, which in turn regulates the abundance of the receptor, and glutamate-dependent behavior of the animals. Ubiquitinated GLR-1 that is not processed by the USP-46 complex is degraded at the lysosome. Impeding lysosomal degradation by expression of a dominant negative enzyme (VPS-4), or perturbing ubiquitin homeostasis through over-expression of ubiquitin itself, reduces or enhances GLR-1 degradation, respectively. Based on our experience with the GLR-1 model system, we are employing cell-specific expression of these known modulators of ubiquitin-mediated degradation to disrupt behaviors that depend on small-molecule receptors in individual sensory neurons. These studies are ongoing, and will shed light on the protein-regulatory networks involved in modulating the abundance and trafficking of cell-surface receptors in diverse cell types.

P1298

Board Number: B641

Autophagy affects glutamate receptor localization at the *Drosophila* neuromuscular junction.

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Glutamate, one of the most abundant neurotransmitters, is responsible for fast excitatory neurotransmission. Glutamate receptors (GluRs) have an important role in learning and memory and

synaptic plasticity can occur through the insertion or removal of postsynaptic glutamate receptors. We have found that autophagy-specific genes (*atg*) influence the localization of glutamate receptors at the *Drosophila* neuromuscular junction. Mutations of *atg1* and *atg8a* result in a significant decrease in postsynaptic GluRs, while cellular levels of GluR protein remain unaffected. Rescue experiments indicate *atg1* is required in neurons and muscle cells, while restoration of *atg1* in glial cells did not rescue GluR localization. To determine the mechanism by which the Atg proteins regulate GluR localization, we are assessing if pharmacological inhibition of autophagy affects GluR localization. In addition, we are trying to determine if inhibition of autophagy affects presynaptic glutamate metabolism thereby altering postsynaptic GluR localization. The results from these experiments will give further insight as to autophagy's role in glutamate receptor trafficking and localization.

P1299

Board Number: B642

Investigation of the active zone protein SYD-2 Liprin α as a substrate of the Anaphase-promoting complex ubiquitin ligase at the *C. elegans* Neuromuscular Junction.

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Ubiquitin-mediated regulation of neuronal protein activity and abundance is critical for proper nervous system development and function. Misregulation of the ubiquitin enzyme system is observed in many neurological disorders where defects in synaptic transmission occur. While several ubiquitin ligases that control the levels and functions of synaptic proteins have been identified, the substrate specificities of many remain unknown. The Anaphase-promoting complex (APC) is a conserved, multi-subunit ubiquitin ligase that controls protein abundance during the cell division cycle. In neurons, the APC regulates post-mitotic functions ranging from axon outgrowth to cell survival and controls glutamatergic synapse formation and synaptic transmission in flies, worms, and mammals, where it is implicated in synaptic plasticity¹. We recently showed the APC also acts to control the balance of excitatory cholinergic to inhibitory GABAergic signaling (E:I balance) at the neuromuscular junction (NMJ) in *Caenorhabditis elegans*². We found the APC prevents excessive muscle excitation by acting in presynaptic inhibitory motor neurons to promote GABA release. Here we report the initial characterization of SYD-2 Liprin α as a potential substrate of the APC at the *C. elegans* NMJ. SYD-2 Liprin α is an active zone protein required for synapse development and function across phylogeny and has been implicated as an APC substrate in fly and mammalian neurons^{3,4}. In *C. elegans*, SYD-2 Liprin α acts presynaptically to promote normal GABA synapse development and NMJ function^{5,6}. SYD-2 Liprin α contains a conserved APC recognition motif (D-box), supporting its potential regulation by the APC. We found a GFP-tagged version of SYD-2 Liprin α accumulates at inhibitory NMJ presynapses in APC loss of function *C. elegans* mutants. Behavior assays with multiple loss of function alleles confirmed the requirement for SYD-2 Liprin α in promoting muscle contraction. Additional behavior experiments testing double loss of function mutants demonstrated loss of SYD-2 Liprin α can partially suppress the increased muscle contraction of APC mutants, suggesting

SYD-2 Liprin α may be one of multiple APC substrates at the NMJ. Mutagenesis studies assessing a requirement for the D-box in the ability of SYD-2 Liprin α to be controlled by the APC are ongoing. Future experiments will determine the effect of an altered APC recognition site on SYD-2 abundance and ubiquitination. 1) Puram SV and Bonni A. 2011. *Sem Cell Molec Biol.* 22:586-594, 2) Kowalski JR *et al. Molec Cell Neurosci.* 2014. 58:62-75, 3) Van Roessel P *et al.* 2004. *Cell* 119:707–718, 4) Hoogenraad CC *et al.* 2007. *Dev Cell.* 12:587-602, 5) Zhen M and Jin Y. 1999. *Nature.* 401:371–375, 6) Sieburth D *et al* 2005. *Nature.* 436:510-516.

P1300

Board Number: B643

Inhibition of the reelin signaling pathway promotes dendritogenesis in mature hippocampal neurons.

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Introduction: Reelin is a protein of the extracellular matrix that is secreted by a subset of GABAergic neurons. By binding to the lipoproteins receptors ApoER2 and VLDLR, reelin regulates intracellular signaling modulating hereby several forms of neuronal plasticity. Here we studied how interference of reelin-mediated signaling in mature hippocampal neurons impacts dendritogenesis and the distribution of the synaptic proteins like NMDA receptor subunits (NR2B and NR2A), the scaffolding protein postsynaptic density 95 (PSD95) and the presynaptic proteins Bassoon and synapsin-I. We hypothesized that blocking of reelin signaling favors an immature state of dendritic tree allowing the presence of immature synapses containing NR2B and SAP102 preferentially. Material and Methods: Expression of reelin was analyzed by immunostaining during development of hippocampal neurons (0-20 days in vitro (DIV)). Mature hippocampal neurons (15DIV) were transfected with GFP alone or with ApoER2 Δ C by magnetofection and chronically treated with GST-RAP or CR-50 to prevent reelin-ApoER2 interactions. At 20 DIV, cultures were fixed with PFA. We analyzed the dendritic architecture and the expression of synaptic proteins using double immunostainings.

Results: With hippocampal maturation, gradually more neurons showed strong reelin immunoreactivity. We also found that exposure of cultures to either CR50 or GST-RAP leads to a significant increase in dendritogenesis relative to control neurons. Application of CR50 also significantly enhanced total dendritic outgrowth. Dendritogenesis was associated with an increment NR2B subunit and with a reduction in PSD95 clustering. Discussion: Our results show that blockage of reelin signaling in mature hippocampal neurons modify the expression of key synaptic proteins at the postsynaptic membrane and promotes dendritogenesis. Our evidence indicates that reelin maintains NR2A-NMDARs at the synapse by interacting with PSD95, occluding hereby the insertion of the NR2B-NMDAR and therefore limiting the dendritogenesis.

P1301**Board Number: B644****DBH and Copper-transporter ATP7B are Regulated by Neurotransmitters in SH-SY5Y Cells.**K. Schmidt¹, S. Lutsenko¹;¹Physiology, Johns Hopkins University School of Medicine, Baltimore, MD

Copper homeostasis plays an important role in the brain since it enables function of copper dependent enzymes such as dopamine-beta-hydroxylase (DBH). DBH is crucial for metabolism of catecholamines and function by converting dopamine into norepinephrine. Mice and human that are deficient in copper transporting ATPases (Cu-ATPases) ATP7A and ATP7B display abnormal levels of catecholamines implying an involvement of Cu-ATPases in DBH metabolism. We have examined whether catecholamines co-regulate Cu-ATPases and DBH in SHSY-5Y cells. We show that dopamine treatment induces trafficking of ATP7B from its basal localization in TGN to the plasma membrane and neurite extensions of differentiated SH-SY5Y cells, whereas ATP7A is not affected by this treatment. Norepinephrine also induced relocation of ATP7B away from the TGN, possibly to vesicles, and increased the amount of DBH secreted into the medium. The time course experiment shows that DBH secretion upon norepinephrine treatment occurs within 1-2 hours. This relatively short time suggests that norepinephrine induced DBH secretion may involve an intracellular signaling mechanism. In order to measure DBH activity, we applied a coupled enzymatic activity assay and measured conversion of tyramine, a surrogate substrate for DBH, para-hydroxybenzaldehyde. Measurement from culture medium of SH-SY5Y cells showed that increased DBH secretion level upon norepinephrine treatment correlated with increased DBH activity. Altogether, our findings indicate that norepinephrine specifically regulates Cu-transporter ATP7B and DBH in the absence of elevated copper thus demonstrating novel mode of regulation of cuproenzymes within the secretory pathway.

P1302**Board Number: B645****Masparidin's Interactions with Signaling Proteins.**L.E. Bailey¹, L.P. Lyman-Henley¹, L.J. Dangott², L.F. Lemanski¹;¹Biological Environmental Sciences, Texas AM University-Commerce, Commerce, TX, ²Biochemistry Biophysics, Texas AM University, College Station, TX

The hereditary spastic paraplegias (HSPs) are a group of upper motor neuron disorders characterized by continuous degradation of efferent neurons in the corticospinal tract. Mast syndrome (SPG21) is an autosomal-recessive complicated form of HSP that originates from a mutation of the ACP33/masparidin gene. Bone Morphogenetic Proteins (BMPs) are multifunctional growth factors that, along with forming bone and cartilage, influence axonal synaptic growth and function. Previous studies have shown multiple HSP-associated proteins to be inhibitors of BMP signaling. Thus, we propose that masparidin is involved in BMP signaling as an additional BMP inhibitor. In fact, previous results have demonstrated

that neurons that are depleted of maspardin exhibit increased axonal branching. My working hypothesis is that maspardin is indispensable in regulating BMP signaling via protein trafficking and degradation. Levels of phosphorylated Smad 1/5 were examined in mouse embryonic fibroblasts (MEFs) by western blot analysis and compared to total Smad levels after stimulation with BMP4 ligand. Comparable experiments were performed in primary neuron cultures from wildtype and knockout mice. Pixel densities were used to compare PSmad/Smad ratios in western blots, as well as confirm quantities of protein present. Results were statistically analyzed via unpaired two-tailed t tests. Increases in PSmad 1/5 signaling were insignificant in wildtype stimulated neurons when compared to controls ($P=0.0911$). In contrast, knockout neurons demonstrated significant increases in levels of PSmad 1/5 in stimulated cells versus controls ($P=0.0462$; $*P<0.05$). Significant increases were also observed between stimulated and control samples in knockout MEFs ($P=0.0466$; $*P<0.05$). These increases were similarly found to be insignificant in wildtype cells ($P=0.6061$). Further investigation is needed to determine the exact mechanism by which maspardin regulates these molecules. Focus has currently expanded to explore other molecules with which maspardin might be involved as a regulator or promoter of function. 6 month and 15 month old knockout and wildtype mouse brains were extracted and subjected to 2D-DIGE analysis. Preliminary results have found a few spots of interest that will be submitted for protein identification using Liquid Chromatography–Tandem Mass Spectrometry (LC-MS/MS) analysis. This is the first investigation of this type in the SPG21 $-/-$ mouse. Because of maspardin's ubiquitous nature, elucidation of proteins with which maspardin may be interacting could be useful in furthering our understanding of other neuronal signaling pathways.

P1303

Board Number: B646

Influence of Protein Size and Binding During Diffusion within the Postsynaptic Density.

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Mechanisms that regulate the insertion, movement, positioning, and removal of glutamate receptors determine the strength of neurotransmission at excitatory synapses. Two key processes that control synaptic receptor number are diffusion within the synaptic and extrasynaptic space and interactions between receptors and scaffold proteins within the postsynaptic density (PSD). Electron microscopy suggests that the PSD is highly crowded, potentially limiting the ability of receptors to diffuse and interact with scaffold proteins. However, the contribution of macromolecular crowding to receptor retention remains to be tested systematically. Here, we combine experimental and computational approaches to test the effect of synaptic steric hindrance on receptor mobility and enrichment. To examine the distinct contributions of crowding and receptor-scaffold binding, we developed a

computational model for AMPAR diffusion in the synaptic and extrasynaptic space, which contains immobile obstacles, representing scaffolding, receptor, and adhesion molecules in the PSD. The spatial distribution of scaffold proteins was determined directly from photoactivated localization microscopy measurements that mapped molecular positions with a resolution of ~ 30 nm. The AMPAR/scaffold association and dissociation rates were adjusted by computer simulations to fit single-particle tracking and fluorescence recovery after photobleaching measurements. The model predicts that variation of receptor size has the most influence on the recovery curves while variation of kinetic rates did not significantly alter receptor residence time or mobility. In order to directly address these questions experimentally and to verify model predictions, we used single-molecule tracking and bulk imaging techniques such as fluorescence recovery after photobleaching to quantify the diffusion dynamics of AMPARs and a set of uniquely designed transmembrane (TM) proteins that mimic receptors on living synapses. We find that diffusion of TM proteins is slowed in the synapse even in the absence of binding interactions (from 0.30 ± 0.005 to $0.24 \pm 0.01 \mu\text{m}^2/\text{s}$). Adding a single synaptic binding motif to a small TM protein also slows its diffusion within the synapse (from 0.15 ± 0.008 to $0.11 \pm 0.01 \mu\text{m}^2/\text{s}$), consistent with modeling results. These results suggest that both protein size and binding play important roles in retaining surface-diffusing TM proteins within the excitatory synapse and shed light on the biophysical mechanisms that lead to high density of AMPARs and other transmembrane proteins at synapses.

P1304

Board Number: B647

Size separation of large proteins and protein complexes under native conditions.

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Proteins do not carry out their functions in isolation but mainly through interactions with other proteins. The elucidation of the biological function of protein complexes is an area of intense research efforts but isolation and analysis of the complexes is challenging as they can be very large and unstable. Size exclusion chromatography (SEC) offers separation by size under mild, non-dissociative conditions. Lewy bodies are abnormal inclusions found in nerve cells in patients with Parkinson's disease and constitute mainly of aggregated forms of the protein α -synuclein. The oligomerization of α -synuclein was studied using SEC and Superose™ 6 Increase 3.2/300, a column with separation range up to Mr 5 000 000 for globular proteins. Two different oligomerization reactions of monomeric α -synuclein with different excess of the aldehyde ONE was compared. The separations showed that the material with ratio 1:15 was heterogenic and not oligomerized to the same extent as the material with ratio 1:30. Based on elution position, the size of the oligomerized material was estimated to Mr > 700 000.

P1305**Board Number: B648****Localization of heteromeric $\alpha 3\beta 4$ nicotinic acetylcholine receptors on the cell surface is regulated by N-cadherin homotypic binding and the actin cytoskeleton.**J.L. Bruses^{1,2};¹Natural Sciences, Mercy College, Dobbs Ferry, NY, ²Institute of Cell Biology and Neuroscience (IBCN), Buenos Aires, Argentina

Neuronal nicotinic acetylcholine receptors (nAChR) are widely expressed in the central and peripheral nervous systems and localize at synaptic and extrasynaptic sites of the cell surface. Various mechanisms control nAChR localization within distinct domains of the cell membrane including incorporation into cholesterol-rich membrane microdomains, and association with scaffolding proteins and the actin cytoskeleton. The homotypic cell adhesion molecule N-cadherin is commonly expressed at synaptic contacts and regulates the actin cytoskeleton through interactions of the cytoplasmic domain with β -catenin and p120-catenin. To examine whether N-cadherin homotypic binding regulates the cell surface localization of nAChR, Chinese hamster ovary (CHO)-K1 cells were transfected with N-cadherin and with $\alpha 3$ and $\beta 4$ nAChR subunits C-terminally fused to a myc-tag epitope. Cell surface expression levels of $\alpha 3\beta 4$ -nAChR within cell-cell contacts and contact-free cell membrane were analyzed by confocal microscopy. In cells not expressing N-cadherin, $\alpha 3\beta 4$ -nAChR was found evenly distributed over the entire cell surface including areas of contact between cells ($100 \pm 16.3\%$), while N-cadherin-mediated cell contacts were largely devoid of $\alpha 3\beta 4$ -nAChR ($3.8 \pm 2.2\%$). Expression of truncated N-cadherin ectodomains lacking the β -catenin binding domain or the entire cytoplasmic domain increased the expression levels of $\alpha 3\beta 4$ -nAChR within cell contacts to $78.3 \pm 13.0\%$ and $102.2 \pm 15.2\%$ respectively, indicating that the interaction of N-cadherin with β -catenin and p120-catenin was necessary for regulating $\alpha 3\beta 4$ -nAChR localization. Treatment with inhibitors of actin polymerization (latrunculin A [$10\mu\text{M}$] and cytochalasin D [$2\mu\text{M}$]) did not significantly affect $\alpha 3\beta 4$ -nAChR expression within N-cadherin-mediated cell contacts ($3.8 \pm 2.7\%$ and $8.2 \pm 2.7\%$ respectively). In contrast, treatment with a cell permeable RhoA inhibitor (C3-exotransferase [$1\mu\text{g/ml}$]) or with the ROCK inhibitor Y27632 [$10\mu\text{M}$] resulted in a significant increase in $\alpha 3\beta 4$ -nAChR levels within N-cadherin-mediated cell contacts ($34.9 \pm 7.1\%$ and $81.5 \pm 23.4\%$ respectively), suggesting that the $\alpha 3\beta 4$ -nAChR localization was regulated by RhoA-ROCK activity downstream of N-cadherin binding. Analysis of $\alpha 3\beta 4$ -nAChR localization in polarized Caco-2 cells showed expression exclusively on the apical cell membrane and colocalization with apical F-actin and with the actin nucleator Arp3. These results suggest that actomyosin contractility downstream of N-cadherin homotypic binding regulates the localization of $\alpha 3\beta 4$ -nAChR within the cell membrane through a mechanism involving N-cadherin cytoplasmic domain and the interaction of $\alpha 3\beta 4$ -nAChR with a pool of F-actin.

Lipids and Membrane Microdomains

P1306

Board Number: B650

The leukodystrophy protein FAM126A/Hyccin regulates PI4P synthesis at the plasma membrane.

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Myelin comprises concentric, tightly apposed layers of lipid-rich glial cell plasma membranes that surround, insulate, and trophically support axons in the vertebrate nervous system. Genetic defects in myelin formation and maintenance cause leukodystrophies, a large, heterogeneous group of brain white matter diseases whose mechanistic underpinnings are poorly understood. Hypomyelination and Congenital Cataract (HCC), one of these disorders, is caused by mutations in the FAM126A gene, which encodes FAM126A/hyccin, a protein of previously unknown function. Here, we show that FAM126A regulates the synthesis of phosphatidylinositol 4-phosphate (PI4P), a key determinant of plasma membrane identity. We demonstrate that FAM126A is an intrinsic component of the phosphatidylinositol 4-kinase complex at the plasma membrane, known to comprise PI 4-kinase III α (PI4KIII α) and two non-catalytic subunits, the peripheral membrane protein EFR3 and the putative scaffold TTC7. FAM126A directly binds to TTC7. A crystal structure of the FAM126A–TTC7 complex reveals an almost all- α -helical heterodimer with an unusually large protein-protein interface consistent with a high-affinity interaction and a conserved surface that may mediate binding to the kinase. Functional analysis of fibroblasts from HCC patients reveals reduced plasma membrane PI4P levels relative to controls. We propose that defects in PI4KIII α -mediated PI4P production in oligodendrocytes may be involved in HCC disease pathogenesis. The specialized function of oligodendrocytes in myelin formation is mediated by their property to massively expand their plasma membrane, and this property is highly dependent on the generation of PI4P and its downstream metabolites PI(4,5)P₂ and PI(3,4,5)P₃. We find that FAM126A is highly expressed in oligodendrocytes, and studies of FAM126A knockout mice support a primary role of this protein in such cells. Thus, our results imply a critical role of FAM126A and PI4P generation in supporting myelin formation, a process of essential importance not only in development but also during remyelination following injury, as occurs in multiple sclerosis and other demyelinating diseases.

P1307**Board Number: B651****The tomato defensin TPP3 induces tumour cell lysis through a conserved mechanism involving PIP2 binding and oligomerization.**A. Baxter^{1,2}, V. Richter¹, I.K. Poon^{1,2}, F.T. Lay¹, C. Adda¹, M. Kvensakul¹, M.D. Hulett^{1,2};¹Biochemistry, La Trobe Institute for Molecular Sciences, Melbourne, Australia, ²Hexima Ltd, Melbourne, Australia

Plant defensins are small innate immune peptides that often exhibit broad spectrum antifungal activity. NaD1 is an antifungal Class II defensin of the Solanaceae family that has also recently been shown to exhibit selective cytotoxic activity towards mammalian tumour cells and represents a promising candidate for the development of anti-cancer therapeutics. NaD1 acts through a novel phosphatidylinositol 4,5-bisphosphate (PIP2)-binding mechanism involving PIP2-mediated oligomerization of NaD1 leading to target membrane destabilization. The class II Tomato Pistil Predominant 3 defensin, TPP3, is a related yet poorly characterized homologue of NaD1. Here, we report through several biological and biophysical approaches that TPP3 shares structural and functional traits with NaD1, indicating an evolutionarily conserved lipid-binding and mechanism of action within this class of defensin. TPP3 exhibited comparable anti-tumour activity to NaD1, which was shown to be dependent on target cell membrane PIP2 to elicit efficient membrane lysis. Structural determination by X-ray crystallography revealed striking similarities between TPP3 and NaD1, with TPP3 adopting the lipid-binding 'cationic grip' dimer conformation of NaD1, indicating that TPP3 is likely to form an oligomeric complex in the presence of its ligand, PIP2. These findings contribute to our broadening understanding of Class II solanaceous plant defensins and their therapeutic potential as anticancer molecules.

P1308**Board Number: B652****“Structure of a lipid-bound extended synaptotagmin indicates a role in lipid transfer”.**C.M. Schauder¹, X. Wu², Y. Saheki³, P. Narayanaswamy⁴, F. Torta⁴, M. Wenk⁵, P. De Camilli³, K.M. Reinisch⁶;¹Molecular Biophysics and Biochemistry, Yale University, New Haven, CT, ²Harvard Medical School, Boston, MA, ³Department of Cell Biology, Howard Hughes Medical Institute, Program in Cellular Neuroscience, Neurodegeneration and Repair, Yale University School of Medicine, New Haven, CT,⁴Department of Biological Sciences, National University of Singapore, Singapore, Singapore,⁵Department of Biochemistry, National University of Singapore, Yong Loo Lin School of Medicine,Singapore, Singapore, ⁶Department of Cell Biology, Yale University School of Medicine, New Haven, CT

It has become increasingly apparent that membrane contact sites, regions of close apposition between two distinct membrane components, facilitate lipid exchange. The mechanisms underlying this type of lipid transfer are largely unknown. SMP domains are proposed lipid binding modules that are found only in proteins localized to membrane contact sites. The extended synaptotagmins (E-Syts) are a family of proteins that tether the endoplasmic reticulum (ER) to the plasma membrane at contact sites and contain an N-terminal ER anchor sequence, an SMP domain and three or more C2 domains. Here we report at 2.44 Å resolution the crystal structure of an N-terminal fragment of human E-Syt2, which includes the SMP domain and two adjacent C2 domains (A,B). The C2A and C2B domains interact to form an arch, which likely is not rigidly positioned with respect to the SMP domain in solution. The SMP beta-barrel fold identifies these domains as members of the tubular-lipid-binding (TULIP) superfamily. In E-Syt2, the SMP domain dimerizes to form a 90-Å-long cylinder. A cavity lined almost exclusively with hydrophobic residues runs the length of the cylinder and is connected to solvent via a “seam”. The electron density within the SMP domain cavity revealed the presence of diacylglycerol lipids, which were subsequently identified as glycerophospholipids via mass spectrometry. These results strongly support a role for E-Syt2 in transferring glycerophospholipids between the ER and the PM and with the identification of the SMP domain as a lipid binding domain, have much farther reaching implications.

P1309

Board Number: B653

Plasticity of PI4KIII α interactions at the plasma membrane.

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Plasma membrane PI4P is an important direct regulator of many processes that occur at the plasma membrane and also a biosynthetic precursor of PI(4,5)P₂ and its downstream metabolites. The majority of this PI4P pool is synthesized by an evolutionarily conserved complex, which has as its core the PI 4-kinase PI4KIII α (Stt4 in yeast) and also comprises TTC7 (Ypp1 in yeast) and the peripheral plasma membrane protein EFR3. While EFR3 is implicated in the recruitment of PI4KIII α , the plasma membrane protein Sfk1 was shown to participate in this targeting and activity in yeast. Here we identify a member of the TMEM150 family as a functional homologue of Sfk1 in mammalian cells. We show that TMEM150A localizes at the plasma membrane and associates with the EFR3-PI4KIII α complex, but in a way that is mutually exclusive with the presence of TTC7 in this complex. In addition, we show that overexpression or knockdown of TMEM150A result in significant changes (increase and decrease, respectively) in the rate of PI(4,5)P₂ resynthesis upon its acute PLC-dependent hydrolysis by activation of a GPCR receptor, the M1 muscarinic receptor. Taken together, our data indicate that TMEM150A helps regulate the function of PI4KIII α at the plasma membrane. The property of PI4KIII α to assemble in two different complexes (TTC7-EFR3-PI4KIII α and TMEM150A-EFR3-PI4KIII α) reveals a plasticity of the molecular interactions that control its localization and function.

P1310**Board Number: B654****The Epidermal Growth Factor Receptor regulates phosphoinositide dynamics and clathrin-mediated endocytosis.**R.C. Delos Santos¹, C.N. Antonescu¹, L. Bone¹, S.J. Bautista¹;¹Department of Chemistry and Biology, Ryerson University, Toronto, ON

Clathrin-mediated endocytosis (CME) is the principal mechanism for internalization of diverse cell surface proteins (called cargo) from the cell surface, and thus is an important regulator of the surface proteome. CME initiates with the recruitment of clathrin, the adaptor protein AP-2, and many other proteins to cargo-enriched membrane invaginations termed clathrin-coated pits (CCP). After undergoing a maturation process that remains poorly understood, these CCPs eventually pinch off to form clathrin-coated vesicles (CCV). Although mechanistically well characterized, there is a paucity of knowledge of how CME can be regulated.

Phosphatidylinositol-(4, 5)-bisphosphate (PIP2) is a key regulator of CME, as it acts as a membrane ligand to recruit the adaptor protein AP-2 (and other proteins) to CCPs. The key regulatory role of PIP2 is evinced by the observation that either experimental increases or decreases of PIP2 levels impair transferrin receptor (TfR) internalization, a well-studied cargo receptor of CME (Antonescu et al. 2011 MBoC 22:2588-600). In resting (unstimulated) cells, PIP2 levels are dynamically controlled by synthesis by phosphatidylinositol-5-kinases (PIP5K) and turnover by inositol 5-phosphatases (e.g. Synaptojanin1, Sjn1). Stimulation of cells with ligands of various signaling receptors, such as epidermal growth factor (EGF) to activate the EGF Receptor (EGFR), results in activation of enzymes that alter PIP2 dynamics, namely phospholipase C β 1 (PLC β 1). In addition to activation of signaling molecules, ligand binding to EGFR also elicits CME of the receptor, suggesting that signaling and internalization must be coordinated. How activation of PLC β 1 by EGF stimulation alters PIP2 levels and dynamics and hence may control CME is poorly understood.

To determine how activation of PLC β 1 by EGF stimulation may regulate CME, we used biochemical ligand internalization assays in combination with siRNA gene silencing of PLC β 1. We find that silencing of PLC has no effect on internalization of TfR (a condition in which PLC β 1 is inactive). In stark contrast, silencing of PLC β 1 results in a decrease in EGFR internalization, suggesting that the requirement for PLC β 1 for CME is specific for certain conditions. Interestingly, TfR internalization is inhibited upon PLC β 1 silencing in cells also stimulated with EGF. Using total internal reflection fluorescence microscope (TIRF-M) of cells expressing fluorescently tagged protein components of CME coupled to customized image analysis, we have quantified the dynamic localization of TfR, EGFR, and PLC β 1 vis-a-vis CCPs. Our findings suggest that PLC β 1 activation by EGFR effects broad changes to PIP2 availability and dynamics at the plasma membrane, thereby regulating clathrin-mediated endocytosis.

P1311**Board Number: B655****Differential regulation of phosphatidylinositol 4,5-bisphosphate homeostasis by Nir2 and Nir3 at ER-Plasma membrane junctions.**C. Chang¹, J. Liou¹;¹University of Texas Southwestern Medical Center, Dallas, TX

Phosphatidylinositol (PI) 4,5-bisphosphate (PIP₂) at the inner leaflet of the plasma membrane (PM) governs many cellular processes, including endocytosis, cytoskeleton dynamics, and store-operated Ca²⁺ entry (SOCE). Importantly, hydrolysis of PIP₂ by receptor-activated phospholipase C (PLC) is pivotal to initiate signaling events via the production of inositol 1,4,5-triphosphate (IP₃) and diacylglycerol (DAG). Meanwhile, it is necessary to replenish the depleted PIP₂ to maintain PIP₂-dependent cellular functions and signaling. It has been shown that PIP₂ levels recovered within minutes following receptor-induced hydrolysis but the mechanisms of PIP₂ replenishment remains unclear. In this study, we demonstrate that non-vesicular delivery of PI originating from the ER is important for rapid PIP₂ replenishment at the PM. Two mammalian PI transfer proteins (PITPs) Nir2 and Nir3 sense phosphatidic acid (PA) production from PIP₂ hydrolysis, and translocate to ER-PM junctions to mediate PIP₂ replenishment. The abilities of Nir2 and Nir3 to mediate PIP₂ replenishment are dependent on their translocation to ER-PM junctions and PI in the ER, suggesting PI transfer at ER-PM junctions. With distinct PITP activities and PA sensitivities, Nir2 and Nir3 differentially regulate PIP₂ homeostasis. Altogether, our findings reveal the long-sought mechanism that couples PIP₂ hydrolysis to its replenishment via Nir2 and Nir3 at ER-PM junctions to maintain PIP₂ homeostasis.

P1312**Board Number: B656****Structure function studies of the sterol transport protein, STARD4.**D.B. Iaea^{1,2}, I. Dikiy¹, I. Kiburu¹, D. Eliezer¹, O. Boudker¹, F.R. Maxfield¹;¹Cornell University Weill Cornell Medical College, New York, NY, ²Tri-Institutional Program in Chemical Biology, Weill Cornell Medical College, New York, NY

Significant differences in lipid distribution are maintained between intracellular organelles. Cholesterol comprises ~30% of the lipid molecules in the plasma membrane and is also enriched in the endocytic recycling compartment (ERC). In the endoplasmic reticulum (ER), cholesterol accounts for 5% of the lipid molecules. There is substantial evidence for high rates of non-vesicular sterol transport in cells. The steroidogenic acute regulator-related lipid-transfer (START) domain containing proteins are involved in several pathways of non-vesicular trafficking of sterols. Among the soluble START proteins, STARD4 is expressed in several tissues and has previously been shown to transfer sterol. The mechanism of membrane interaction and sterol binding of STARD4 is unclear. From the crystal structure of STARD4, several positively charged residues that form a basic patch were identified and suggested to facilitate membrane docking through interaction with anionic lipids. To investigate the role of the basic patch, we

ablated the charge of the basic patch and examined the effect by using an in vitro sterol transport assay. Mutation of the basic patch resulted in a 4-7 fold reduction in sterol transport, indicating that this region interacts with anionic membranes and is required for efficient sterol transfer. Recently, the activity of two sterol transport protein were found to be modulated by PI(4)P in a membrane specific manner. This suggested a potential role of phosphatidylinositol phosphates (PIPs) to modulate STARD4 activity. To investigate this potential role, the anionic lipids of either donor or acceptor liposomes were removed and replaced with physiologic levels, 2 mol%, of individual PIPs. Utilizing bulk phosphatidylserine (PS) anionic lipids, STARD4 is able to transfer 7 molecules of sterol/STARD4/minute. We find that PI(4,5)P₂ increases STARD4 activity only in the donor while PI(5)P and PI(3,5)P₂, increases activity only in the acceptor. In each of these membrane specific PIPs, STARD4 transfers 1 molecules of sterol/STARD4/second indicating nearly a 10-fold increase in transfer rate. Additionally, the increase in STARD4 sterol transfer activity is PIPs dose dependent, with sterol transfer rates slow from 0-1mol% while increasing and maintained from 2-10mol%. This suggests that there is a minimal threshold of PIPs required for STARD4 activity to be increased. We have also found that the membrane specific PIP increase in STARD4 sterol transfer is maintained in the presence of bulk PS anionic lipids. Taken together, these studies provide a mechanism for rapid vectorial transport as STARD4 extracts sterol from PI(4,5)P₂ (donor) membranes and delivers to PI(5)P and PI(3,5)P₂ (acceptor) membranes.

P1313

Board Number: B657

The Serine/Threonine kinase Par1b regulates ORP3 localization via 14-3-3 binding.

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Simple epithelial cells have distinct luminal (apical) surfaces separated by tight junctions from the intercellular and blood barrier-interacting (baso-lateral) surface. The maintenance of this apico-basal polarity is crucial for proper functioning tissues such as kidney tubules, liver, and gastric epithelia. Par1b is a serine/threonine kinase which is well known for its role in establishment and maintenance of epithelial polarity. In MDCK cells, loss of Par1b correlates with loss of polarity. Conversely, overexpression promotes the establishment of a hepatic-type polarity, where the apical surface forms between the lateral domain of neighboring cells. The mechanism of how Par1b regulates polarity is unclear; however, preliminary data suggests that its kinase activity is crucial for lumen development and polarity.

We report ORP3, Oxysterol Related binding Protein-3 to be a novel Par1b substrate in MDCK cells. ORP3 has three domains, a PH domain that binds to the plasma membrane (PM), an FFAT motif that binds to VAP (an integral membrane protein of the Endoplasmic Reticulum (ER)), and an ORD domain that is thought to be responsible for oxysterol binding. We identified two Par1b-phosphorylation sites in ORP3, which are both predicted 14-3-3 binding sites. We established that a phospho-deficient ORP3 (SA-ORP3) bound less 14-3-3 than the WT protein. WT-ORP3 localized mostly to the ER and the cytoplasm, while

the SA-ORP3 additionally localized to the nuclear envelope and in punctae surrounding the cell cortex. The localization of WT-ORP3 in Par1b knock down MDCK cells phenocopied the SA-ORP3 localization. Using live cell imaging in MDCK cells, we observed that upon compactation, the punctae appeared more strongly associated with cell-cell contacts. The punctae remained the same size for over 48 hours, regardless of ORP3 expression levels. This suggests that they are unlikely protein aggregates. While disruption of the actin cytoskeleton did not change the punctuated pattern, microtubule disruption induced larger punctae, suggesting that microtubules play a role in maintaining their structure. We hypothesize that 14-3-3 regulation of ORP3 contributes to its localization to membrane contact sites (MCS) at the interface of the PM and ER. Specifically, we predict that the absence of phosphorylation by Par1b, and the resulting lack of 14-3-3 binding better allow the ORP3 to contact the PM.

P1314

Board Number: B658

Differentiation of the Plasma Membrane Phenotype in Mesenchymal Stem Cells.

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The organization of metazoan membranes into functional domains is a key feature of their physiology. These domains, determined by lipid and protein composition and driven by specific intermolecular interactions, spatially organize membrane components to actively regulate signal transduction across the membrane. Despite the functional importance of membrane domains in cellular physiology, the differences in membrane organization between cell types, and how these impact cell-specific signaling, have not been explored. Moreover, despite the central role for lipid composition in membrane organization, the specific lipid repertoires of various cell lineages and their resulting regulation of plasma membrane (PM) organization and functionality remain to be determined. Mesenchymal stem cells (MSCs) are an ideal model system to investigate these questions, as they can be directed to differentiate into several functionally distinct lineages (e.g. adipocyte, osteoblast) with minimal biochemical perturbations under identical culture conditions. We used MSCs to evaluate the differentiation of the plasma membrane phenotype, defined by (1) the detailed, comprehensive lipid composition; (2) membrane structure; and (3) functional signal transduction. We observed dramatic divergence of all three aspects of the PM phenotype during MSC differentiation. Using quantitative comprehensive shotgun mass spectrometry, we determined the PM lipidomes of differentiated MSCs and found that adipocyte lipids are characterized by shorter and more saturated fatty acids than undifferentiated cells, while osteogenic cell lipids have longer fatty acid tails and are more polyunsaturated. These lipidomic rearrangements led to changes in the physical properties of the PMs, with a divergence of both membrane fluidity and the stability of lipid raft domains associated with MSC differentiation. Taken together, these observations elucidate the compositional determinants of biophysical properties in biological membranes, as well as identify lineage-specific PM features. These

features were used as design principles for rational remodeling of the PM phenotype to promote a specific cellular response. Namely, supplementation of MSCs with a lipid upregulated in osteoblasts (the ω -3 poly-unsaturated fatty acid DHA) promoted the osteogenic PM phenotype in undifferentiated MSCs and thereby potentiated osteogenic differentiation via the BMPR pathway. These results comprise the first observations of compositional and structural differentiation of cellular membranes, identify the compositional determinants of biological membrane structure, demonstrate the plasticity of cellular lipidomes, and enable rational engineering of the plasma membrane phenotype to promote MSC osteogenesis.

P1315

Board Number: B659

Switch-like precision of cholesterol-sensing proteins arises due to limited accessibility of membrane cholesterol.

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Cholesterol levels in mammalian cells are maintained within narrow limits by a network of proteins that measure cholesterol concentrations and then regulate its synthesis and uptake. This regulatory protein network resides in the ER membrane where the cholesterol concentration fluctuates around a set-point of 5 mole% of total lipids. The cholesterol sensor in this system is Scap, a transmembrane protein that regulates the transport and subsequent proteolytic activation of SREBP. The sensitivity of Scap as a sensor is sharp and switch-like; a small increase in ER cholesterol halts both synthesis and uptake. The molecular mechanism by which Scap can measure ER cholesterol with such precision is largely unknown. The sigmoidal response of Scap could arise from several mechanisms, including positive cooperativity (protein effects) and limited accessibility of cholesterol (membrane effects). A convenient model for eukaryotic membrane-bound Scap is a family of soluble bacterial toxins that show identical switch-like specificity for endoplasmic reticulum membrane cholesterol. Using this model, we show that sigmoidal responses can arise primarily due to membrane effects. Truncated versions of these toxins fail to form oligomers but still show sigmoidal binding to cholesterol-containing membranes. The non-linear response emerges because interactions between bilayer lipids control cholesterol accessibility to toxins in a threshold-like fashion. Around these thresholds, affinity of toxins for membrane cholesterol varies by >100-fold, generating highly cooperative lipid-dependent responses independent of protein-protein interactions. Such lipid-driven cooperativity may control the sensitivity of many cholesterol-dependent processes.

P1316**Board Number: B660****Structural determinants and functional consequences of transmembrane protein partitioning to membrane rafts.**I. Levental¹;¹Integrative Biology and Pharmacology, University of Texas - Houston Medical School, Houston, TX

The plasma membrane is the physical and functional interface between a cell and its surroundings, and is therefore responsible for a myriad of parallel processing tasks, each of which must be tightly regulated to avoid aberrant signaling. To achieve this complexity, the functionality of the plasma membrane is enhanced by its subdivision into functional lateral domains, including lipid rafts - ordered lipid and protein assemblies whose formation is driven by lipid interactions, and which recruit specific membrane proteins by virtue of their unique physicochemical environment. This selective protein recruitment underlies the functionality of membrane domains; however, the structural determinants of protein partitioning to membrane rafts remain almost wholly unexplored. We have developed and characterized a robust experimental system for isolating and observing intact plasma membranes, allowing the first direct, quantitative measurements of protein affinity for raft domains. Our observations confirm that protein transmembrane domains (TMDs) contain the necessary determinants for raft affinity, as chimeric combinations between TMDs and intra/extracellular protein domains retain the raft affinity of the isolated TMDs. To explore the structural bases for these observations, we tested raft partitioning for a panel of TMD variants and confirmed the long-standing theoretical prediction that longer TMDs would prefer the thicker hydrophobic core of membrane rafts, and thus that TMD length is a key determinant of raft partitioning. We have extended these experimental observations by Molecular Dynamics modeling of protein TMDs in a simulated membrane environment, generating testable predictions for protein-protein and protein-lipid interactions. Using these predictions, we have identified a novel PxxxG motif in transmembrane α -helices that appears to be both necessary and sufficient for raft targeting by the transmembrane domain of a specific immune system adapter protein - Linker for Activation of T-cells (LAT). This motif - related to the common GxxxG motif that mediates helix-helix oligomerization - drives lateral clustering of LAT to promote raft association. We have generalized these findings by bioinformatic approaches to generate predictive heuristics for protein affinity for membrane rafts. Finally, we are exploring the functional consequence of raft affinity, by the raft partitioning of LAT constructs with their efficiency in rescuing activation of leukocytes rendered unresponsive to antigen by a knockdown of endogenous LAT. In summary, our results identify proteins that rely on raft association for their function, define the physicochemical nature of this association, and clarify the mechanisms by which PM organization regulates cell physiology.

P1317**Board Number: B661****Multi-protein assemblies underlie the mesoscale organization of the plasma membrane.**S.K. Saka¹, A. Honigmann², C. Eggeling³, S.W. Hell², T. Lang⁴, S.O. Rizzoli¹;¹Department of Neuro- and Sensory Physiology, University of Göttingen Medical Center, Goettingen, Germany, ²Department of Nanobiophotonics, Max Planck Institute for Biophysical Chemistry, Goettingen, Germany, ³MRC Human Immunology Unit, Weatherall Institute of Molecular Medicine, Oxford, UK, ⁴Department of Membrane Biochemistry, Life and Medical Sciences (LIMES) Institute, Bonn, Germany

Most proteins have uneven distributions in the plasma membrane (Lang and Rizzoli, Physiology, 2010). This may be caused by mechanisms specific to each protein, or may be a consequence of a general pattern that affects the distribution of all membrane proteins. To find out if a general mechanism exists behind this observation, we introduced several imaging approaches that aim to investigate all proteins in the plasma membrane simultaneously rather than focusing on interactions of individual protein species. This was achieved by large-scale metabolic labeling of proteins in mammalian cells through extended incorporation of non-canonical amino acid analogues, followed by fluorescent tagging via click chemistry (Dieterich et al., Nature Neuroscience, 2010). By combining this direct labeling method with super-resolution stimulated emission depletion (STED) microscopy, we studied the protein patterning in plasma membranes of living cells, as well as in membrane sheets. We found that a general mosaic-like pattern governs the proteins organization (Saka et al., Nature Communications, 2014). Multiple proteins were heterogeneously gathered into protein-rich domains surrounded by a protein-poor background. We termed these long-lived high-abundance domains “protein assemblies” and examined the contributions of different factors to their formation and maintenance. We identified cholesterol as the main organizer of the assembly pattern and the actin cytoskeleton as a secondary factor that borders and separates the assemblies. To understand the relation of this mesoscale arrangement to the nanoclusters of individual protein species, we analyzed distributions of specific proteins with respect to the protein assemblies. All of the specific proteins we analyzed were enriched in the assemblies, but they displayed differential enrichment profiles. Many proteins were preferentially located in particular areas within the assemblies, such as their edges or centers. Functionally related protein groups showed similar preferences, suggesting that functional protein-protein interactions create specialized subdomains within the assemblies. We conclude that the assemblies constitute a fundamental principle of the mesoscale membrane organization, which affects the nanoscale patterning of most membrane proteins, and possibly also their activity.

P1318**Board Number: B662****Transbilayer coupling via long acyl chains and immobilized phosphatidylserine mediate actin-based nanoclusters of outer leaflet lipids.**A.A. Anilkumar¹, R. Raghupathy¹, A. Polley², R. Vishwakarma³, Z. Guo⁴, M. Rao^{2,5}, S. Mayor⁵;¹Cellular organisation and signalling, National Centre for Biological Sciences, Bangalore, India, ²Raman Research Institute, Bangalore, India, ³Indian Institute of Integrative Medicine, Bangalore, India,⁴Department of Chemistry, Wayne State Univ, Detroit, MI, ⁵National Centre for Biological Sciences (NCBS), TIFR, Bangalore, India

A significant percentage (10%) of eukaryotic membrane proteins are attached to GPI anchors which help them attach to the outer leaflet of the plasma membrane. The significance of the GPI anchor lies in the fact that it helps to localize these molecules to cholesterol and sphingolipid enriched domains. Work over several years from our laboratory has shown that a dynamic actin cytoskeleton plays a major role in the organization of GPI-APs into cholesterol-sensitive nanoclusters (Gowrishankar et al., 2012). A key question is how GPI-APs at the outer leaflet of the membrane bilayer couples to the actin cytoskeleton at the inner leaflet. A likely possibility is that dynamic actin filaments couple to the inner leaflet lipids either directly or indirectly, which in turn are connected to the outer leaflet via a transbilayer coupling mechanism involving long acyl chain lipids both in the GPI-anchors and the coupling lipid species at the inner leaflet. Phosphatidylserine (PS) which is known to interact with a number of actin binding proteins is the prime candidate for such a lipid species. We also hypothesize that these microdomains could lead to the local ordering of the membrane which could act as sorting platforms for a number of signaling molecules.

We study nanocluster organization of GPI-APs at the cell surface by FRET (specifically homo-FRET) between fluorescently labeled GPI-APs. We then make perturbations in lipid structure of the GPI-anchor by manipulating their acyl chains of GPI-AP by utilizing mutations in enzymes that have a role in fatty acid remodeling. We have also explored the inner leaflet linker that facilitates the transbilayer coupling by exogeneously adding different lipid species of varying chain lengths in cells deficient for PS biosynthesis. Atomistic molecular dynamic simulations of asymmetric, multicomponent membrane bilayers have also been used to explore the role of long acyl chain in the coupling of the inner and outer leaflet lipids. Our results suggest that long-saturated acyl chains in the GPI-anchor, and in PS at the inner leaflet lipid with adequate amount of cholesterol are necessary ingredients for nanocluster formation. These results provide evidence for a transbilayer link between the outer leaflet and the inner-leaflet required for GPI-AP nanoclustering.

References

Gowrishankar, K., Ghosh, S., Saha, S., C, R., Mayor, S., and Rao, M. (2012). Active remodeling of cortical actin regulates spatiotemporal organization of cell surface molecules. *Cell* 149, 1353–1367.

P1319**Board Number: B663****Clustering of lipid rafts is accelerated by active coagulation factor IX via exposure to phosphatidylserine at the adhesion complex.**H. Kitano¹, A. Mamiya¹, T. Ishikawa¹, Y. Fujiwara¹, M. Yoshida¹, S. Kokubun¹, C. Hidai¹;¹Nihon Univ Sch Med, Tokyo, Japan

Introduction: Lipid rafts provide entrance for many types of materials. Recently, we reported that an EGF domain in active coagulation factor IX (EGF-F9) increases lipid raft formation and accelerates cell migration. However, the details of the mechanism are not well understood. **Materials & Methods:** A431 cells derived from human squamous cell carcinoma were treated with recombinant EGF-F9. Cells were immunostained and labeled with probes for lipid rafts or phosphatidylserine (PS) and observed with confocal or TIRF microscopy. **Results:** A431 cells adhered to the matrix at the bottom of the cell and the tips of filopodia via an adhesion complex consisting of integrin $\alpha 3\beta 1$ and paxillin. After treatment with EGF-F9 for 3 minutes, cholera toxin subunit B (CTxB) binding domains emerged at the adhesive tips of filopodia, and CTxB then stained the shafts of the filopodia. Finally, large clusters of CTxB domains were observed at the edge of the cell body. Markers for lipid rafts including caveolin-1 and a GPI-anchored protein colocalized with CTxB. Annexin V and p-SIVA staining revealed that PS was exposed at the tips of filopodia, flowed on shafts of the filopodia, and colocalized with CTxB in rafts. Immunocytochemistry showed that scramblase-1 protein was present at the tips of filopodia. PS exposure and lipid raft clustering induced by EGF-F9 were suppressed in the presence of Ca⁺⁺-free buffer or siRNA for scramblase-1, and thus, lipid raft clustering appears to depend on PS exposure by scramblase-1. **Discussion:** Active F9 accelerated PS exposure around adhesion complexes at filopodia and induced clustering of lipid rafts in the cell body. PS exposure is thought to be observed in cells undergoing apoptosis. However, PS exposure by active F9 was observed within 5 minutes of the stimulus with clustering of lipid rafts and appears to be involved in apoptosis. Because lipid rafts are involved in multiple cell signaling events, clustering may cause rapid and comprehensive changes in cell signaling. Because the EGF-F9 motif is present in other proteins, the function of the motif in signal transduction should be further studied.

P1320**Board Number: B664****Probing the dynamics of raft lipids induced by receptor-mediated signaling in living cells.**C. Hsieh¹, Y.R. Lin², C. Chou³;¹Institute of Physics, Academia Sinica, Taipei, Taiwan, ²Department of Chemistry, National Taiwan University, Taipei, Taiwan, ³Academia Sinica, Taipei, Taiwan

Lipid rafts, the relatively ordered and tightly packed membrane microdomains with enriched cholesterol and glycosphingolipids, play an important role in compartmentalizing cellular processes through the assembling of signaling molecules and regulating membrane protein trafficking. However, the dynamics of lipid rafts involving signaling pathways remain elusive. There are several issues in studying the dynamics of lipid raft. Firstly, most studies regarding lipid rafts used proteins as biomarkers instead of using lipid molecules. Secondly, multivalent toxins used for labeling lipids are also commonly applied despite they may cause some aggregation of lipids. Thirdly, it is rather challenging to observe the dynamics of lipid rafts induced by receptor-mediated process since the whole signaling process happens within only a few minutes. In this study, we investigated the dynamics of lipid rafts through fluorescence imaging by conjugating single fluorescent dyes to the lipid molecules. Sphingomyelin was used as the lipid raft marker while phosphoglycerolipid as the non-lipid raft marker. The whole process of signaling dynamics was slowed down from few minutes to hours by controlling the ligand-binding area to be within tens of nanometers. The movements of receptors were also recorded with nano-sized fluorescent bead. The raft lipids not only diffuse in and out the ligand-receptor binding area, but also aggregate in the raft domain while moving along with the receptors on cell membrane. During the co-migrating process of lipid raft with the receptors, the concentration of raft lipids dramatically increases 2.8-fold in an averaged area of $1 \mu\text{m}^2$. The increment in the concentration of raft lipids in a fixed area of raft domain correlates to a slower diffusion of the receptors. The diffusion coefficients of receptors are determined to be varying from 0.004 to $0.5 \mu\text{m}^2/\text{s}$ during the raft lipid aggregation process. To our knowledge, our study presents the first direct observation of the dynamics of lipid raft by following the early events of raft lipid aggregation induced by receptor-mediated signaling in a living cell.

P1321**Board Number: B665****Phosphorylation Control Coordinates Very Long Chain Fatty Acid Synthesis.**D.K. Olson¹, F. Fröhlich¹, R. Christiano¹, H. Hannibal-Bac², C. Ejsing², T. Walther¹;¹Genetics and Complex Diseases, Harvard School of Public Health, Boston, MA, ²Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark

Eukaryotic membranes are composed primarily of lipids from three classes: glycerophospholipids, sterols and sphingolipids. Sphingolipids are particularly abundant in the plasma membrane, sealing cells from the environment. They are also important for membrane trafficking/ organization and as

messenger molecules. Sphingolipids are de novo synthesized from a very long chain fatty acid (VLCFA) and a sphingoid long chain base that are amide-linked to form ceramide, to which various head-groups are added. Little is known concerning the regulation of sphingolipid levels or the coordination of VLCFA levels with the availability of other sphingolipid synthesis intermediates. Here we show that this coordination is achieved by regulation of VLCFA synthesis through phosphorylation of Fen1, a key enzyme of the fatty acid elongation cycle in *Saccharomyces cerevisiae*. In addition, we show Fen1 phosphorylation to be dependent upon Rom2, a nucleotide exchange factor responsible for activation of Rho1. Our data reveal a key regulation point to maintain homeostasis between different sphingolipid metabolic branches. They also suggest that multiple signal transduction pathways from the plasma membrane cooperate to regulate lipid metabolism for membrane homeostasis.

P1322

Board Number: B666

On the symbiotic origins of eukaryotic plasma membranes.

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Formation of the cellular plasma membrane (boundary), driven by self aggregation of amphipathic molecules, was a crucial step in the origin of life. However, as compared to numerous sequence-structure-function studies on proteins and nucleic acids, literature on assembly of biological membranes from their major constituents (i.e. lipids) to form a variety of functional structures (including boundaries) is limited. In an earlier study we reported extraction of curvature preferences of individual lipids from molecular dynamic simulation data of several “flat” bilayers [1]. In spite of the “flatness” of the systems analyzed, we found curvature preferences exhibited by individual lipid components indicating possible mechanisms (at thermodynamic and kinetic levels) of asymmetric distribution of single-lipid molecules with specific shape parameters leading to segregated domains/phases, and possibly nanoscale curvatures even in the homogenous pure bilayer systems. In this work, we focus on possible origins of membrane bilayers as boundaries of living cells. Inspired by sequence-structure-function studies on nucleic acids and proteins, we analyzed ~3000 plasma membrane lipid constituents of 263 cellular species in the three life domains (archaea, eubacteria and eukaryotes). For membrane lipids common to plasma membranes of the three domains, number of carbon atoms in eubacteria was found to be higher than those in eukaryotes. However, mutually exclusive subsets of same data show exactly the opposite - number of carbon atoms in lipids of eukaryotes was higher than eubacteria. This remarkable statistical paradox, called Simpson’s paradox, was found to be absent for lipids in archaea and for lipids not common to plasma membranes of the three domains. This indicates presence of interaction(s) and/or association(s) in lipids forming plasma membranes of eubacteria and eukaryotes, but not for those in archaea. Further exploration of lipid structures, responsible for physico-chemical properties of plasma membranes, leads to the inference that the first compartmentalized cells, i.e. eukaryotic single cells, had symbiotic origins with emphasis on eubacterial components as a source of their plasma membranes. Thus, here we report the first evidence (to our knowledge) on symbiotic origins of eukaryotic cells, with

a larger role of eubacteria in formation of plasma membranes, based on the “third front” (i.e. lipids), in addition to the growing compositional data from nucleic acids and proteins.

Reference: [1] Bansal S, and Mittal A (2013). Extracting curvature preferences of lipids assembled in flat bilayers shows possible kinetic windows for genesis of bilayer asymmetry and domain formation in biological membranes. *J Membr Biol* 246, 557-570.

P1323

Board Number: B667

Cell detachment induces Lyn and Fyn activation through the change in their membrane distribution.

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Dissociation of cell-matrix interaction normally induces apoptosis in epithelial cells. However, metastatic cancer cells can survive in suspension after detachment from the primary tissue. The Src-family, a family of non-receptor tyrosine kinases, is involved in cell survival, transformation and metastasis. Each member of Src-family kinases is located on the cytoplasmic side of cellular membranes via posttranslational lipid modification. To examine the regulation mechanism of the activity of Src-family kinases in suspension cells, we focused on the interplay between the kinase activity and the membrane distribution of Src-family kinases.

For the analysis of the membrane distribution of Src-family kinases, we used sucrose density gradient fractionation without detergent. We found that the main peak of the membrane distributions of palmitoylated members of Src-family kinases, such as Lyn and Fyn, were changed upon cell detachment from the low-density fractions to the high-density fractions. Cell detachment induces both cholesterol depletion in the plasma membrane and dynamin-dependent internalization of caveolae. Each main peak of the membrane distributions of Lyn and Fyn in suspension cells treated with cholesterol and the dynamin inhibitor dynasore was located in the low-density fractions similar to that in adherent cells. These results may suggest that the changes in the membrane distributions of Lyn and Fyn upon cell detachment involve cholesterol depletion and dynamin activity.

The autophosphorylation levels of Lyn and Fyn and the in vitro kinase assay of Lyn showed that the activities of Lyn and Fyn were increased upon cell detachment. Furthermore, Lyn activity in the high-density fraction was higher than that in the low-density fraction in adherent cells. Therefore, we hypothesized that the change in the membrane distribution of Lyn involves the activation of Lyn upon cell detachment. The incorporation of cholesterol into the cells in suspension interfered with not only the change in the membrane distribution of Lyn but also the kinase activation of Lyn in suspension cells. These results suggest that the cholesterol depletion in the plasma membrane may cause both the change in the membrane distribution of Lyn and the kinase activation of Lyn in suspension cells.

P1324**Board Number: B668****Peripheral nervous system plasmalogens regulate Schwann cell differentiation and myelination.**T. Ferreira da Silva^{1,2}, J. Eira¹, A. Lopes¹, A. Malheiro¹, V. Sousa^{1,2}, M. Sousa¹, P. Brites¹;¹Nerve Regeneration, Instituto de Biologia Molecular e Celular - IBMC, Porto, Portugal, ²Instituto de Ciências Biomédicas Abel Salazar - ICBAS, Porto, Portugal

Schwann cells, the myelinating glia in the peripheral nervous system (PNS) undergo several morphological and biochemical changes that are required for membrane outgrowth and wrapping of individual axons. Myelin is highly enriched in plasmalogens, a class of ether-phospholipids, which may account for 80% of the total glycerophosphoethanolamine pool. The importance of ether-phospholipids is highlighted by the severe clinical presentation of rhizomelic chondrodysplasia punctata (RCDP), a disorder caused by impairment in the plasmalogens biosynthesis. To investigate the role of these ether-phospholipids in Schwann cell biology and myelination, we examined two mouse models with complete impairments in their biosynthesis, i.e., the Pex7 and Gnpat knockout (KO) mice. The histological characterization of sciatic nerves from KO mice revealed impaired radial sorting and myelination. Impaired myelination was also evident in in vitro cultures of embryonic dorsal root ganglia from Gnpat KO mice, and following sciatic nerve crush. Myelin devoid of plasmalogens was less compact and showed abnormal appositions. Myelin compaction in the absence of plasmalogens was partially accomplished through the action of myelin-basic protein (MBP), as a combined defect in plasmalogens and MBP severely affected myelination. These changes in myelin and Schwann cell morphology ultimately lead to an adult-onset severe demyelination with axonal loss. A deficiency in plasmalogens impairs AKT-mediated signaling, causing a dysregulation in GSK3 β and modulates the defects observed in Schwann cell differentiation and myelination. In summary, our findings reveal the pivotal role of plasmalogens for myelination and highlight the importance of these phospholipids during neurodegeneration.

P1325**Board Number: B669****Rab1B role in LDs metabolism.**I. Nevo-Yassaf¹, Y. Yaffe², M. Lovelle¹, E.H. Sklan³, K. Hirschberg¹;¹Pathology, Tel Aviv University, Tel Aviv, Israel, ²Department of Cell and Developmental Biology, Tel Aviv University, Tel Aviv, Israel, ³Clinical Microbiology and Immunology, Tel Aviv University, Tel Aviv, Israel

Lipid droplets (LDs) are the principal organelles for the storage of cholesterol esters and neutral lipids in eukaryotes. Considered for a long time as mere inert deposits, LDs are now conceived as dynamic organelles implicated in numerous biological processes in health and disease. Despite the progress in lipid-droplets research, the molecular mechanisms that underlie LD formation, growth and movement as

well as interaction with other organelles remain for the most part unclear. Here we show for the first time that Rab1B protein, a small GTPase known for its role in ER-to-Golgi trafficking and Golgi biogenesis, has a role in LD metabolism. Our previously published data demonstrate that Rab1B GTPase activity is essential for the integrity of HCV LDs-associated viral replication complexes. Using live cell imaging we found that Rab1B is localized to LDs. Next, we asked whether Rab1 activity is associated with LD metabolism. Overexpression of Rab1B-WT and Rab1B constitutively active (Rab1B-CA) mutant resulted in a decrease in LDs size accompanied by a lower Bodipy staining intensity. Cells expressing Rab1B dominant negative (Rab1B-DN) mutant abolished all steady state LDs. Noteworthy is the fact that in the presence of Rab1DN the Bodipy stain strongly labeled ER membranes, suggesting that lipid synthesis was not affected. Affinity pull-down mass spectrometry analysis in Huh-7 cell line demonstrated that Rab1B interacts with flotillin-1, a protein that is localized to LDs upon oleic acid (OA) treatment and may have a role in lipid regulation or trafficking towards these organelles. Confocal images of live cells demonstrate co-localization of flotillin-1 and Rab1B-WT as well as Rab1B-CA. This co-localization was not seen for flotillin-1 and the DN mutant of Rab1B. Our results support that Rab1B activity is a prerequisite for LD metabolism. We hypothesize that Rab1B is responsible for the targeting of specific enzymes associated with triglycerides storage and catabolism.

P1326

Board Number: B670

Galectin-1 is critical for the structural integrity of mast cells.

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Gangliosides are active components of the outer leaflet of the plasma membrane of eukaryotic cells that jointly with cholesterol, sphingolipids and proteins with lipidic modifications are major constituents of lipid rafts. Lipid raft components can act as receptors for viruses and bacterial toxins as well as modulating receptor functions in several cell types, including mast cells. Because of the importance of gangliosides in mast cell biology, the aim of this study was to compare the protein profile of the rat mast cell line, RBL-2H3, with the RBL-2H3 mutant cell line D1 that is deficient in GM1 and the gangliosides derived from GD1b. A comparative proteomic analysis of total cell lysates from the two cell lines was done using 2D-Gel electrophoresis, gel image analysis and mass spectrometry. In addition to fourteen proteins differentially expressed between the cells lines, it was found that galectin-1 is expressed only in the wild type RBL-2H3 cells and not in the D1 cells. However, RT-PCR results showed that D1 cells do have transcripts for galectin-1. Galectins belong to a subgroup of animal lectins and are characterized by a carbohydrate recognition domain (CRD). In other cell types, several studies have shown the importance of galectin-1 in the regulation of cell adhesion, growth, immune response, and in lipid raft reorganization. In order to study the effects of galectin-1 on lipid raft organization, cellular morphology and degranulation in mast cells, both cell lines were transfected with EGFP-galectin-1. By scanning electron microscopy, the expression of galectin-1 in deficient cells led to fewer ruffles on the surface,

indicating that the absence of galectin-1 is reflected in plasma membrane alterations. By immunofluorescence, in the transfected D1 cells the microtubules were organized in a typical radial distribution, different from the D1 cells whose microtubules were organized circumferentially in a basket shape. In cells transfected with galectin-1 and stimulated via FcεRI, the receptor and LAT can be found in the lipid raft fractions, in contrast to the untransfected D1 cells. However, galectin-1 transfection was not sufficient to restore mast cell degranulation via FcεRI. These results show that galectin-1 has a role in preserving the structure of mast cells, which may be related to its function in plasma membrane organization.

P1327

Board Number: B671

Mast cell specific gangliosides modulate NFAT and NFκB activation in RBL-2H3 cells.

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Mast cells are immunoregulatory cells that participate in diverse biological events. The action of mast cells is directly related to their activation and subsequent mediator release. Early signal transduction events occur in lipid rafts in the plasma membrane. In rodent mast cells, GD1b-derived gangliosides are known constituents of lipid rafts. The cross-linking of these gangliosides by mAb AA4 promotes a partial activation of mast cells, similar to that observed by activation via FcεRI. Since it is known that stimulation via FcεRI activates the transcription factors NFAT and NFκB, the ability of the cross-linked GD1b-derived gangliosides to activate NFAT and NFκB was investigated. The GFP-reporter cell lines VB9 (NFAT) and NFκB₂ were used to assess activation. The cells were stimulated for 1 h with mAb AA4, rinsed and incubated for 5h (NFκB) or 15 h (NFAT). As a positive control, IgE-sensitized cells were rinsed and stimulated with antigen (DNP₄₈-HSA; Sigma Aldrich) for 6 (NFκB) or 16 h (NFAT). The percentage of GFP positive cells was determined using a Guava Easy Cyte Mini System and the data analyzed using Cytosoft Blue (Guava Technologies). Cross-linking the GD1b-derived gangliosides activated both NFAT (35% GFP positive cells stimulated with 10 µg/mL mAb AA4) and NFκB (38% GFP positive cells stimulated with 5 µg/mL mAb AA4) in comparison with 91% (NFAT) and 57% (NFκB) in the positive controls. However, using GFP-reporter cell lines deficient in Syk, crosslinking of the gangliosides derived from GD1b did not activate either NFAT or NFκB. Proteomic analysis of the lipid rafts showed that when the gangliosides were aggregated by mAb AA4, the protein composition is altered in comparison with cells not exposed to mAb AA4. An understanding of the role of gangliosides in mast cell activation may lead to new therapeutic targets for allergy and inflammation.

P1328**Board Number: B672****Scaling up through teamwork: Ankyrin-G microdomains control large-scale cellular patterning through opposing endocytosis.**P.M. Jenkins¹, M. He², W. Tseng², V. Bennett^{1,3};¹Department of Biochemistry, Duke University, Durham, NC, ²Pharmacology and Cancer Biology, Duke University, Durham, NC, ³Howard Hughes Medical Institute, Duke University, Durham, NC

Spectrin and ankyrin are plasma membrane-associated proteins that are localized to discrete microdomains within the plane of the lateral membrane of columnar epithelia (1), yet they control biogenesis and identity of the entire lateral membrane. It remains unknown how these microdomains, which only cover ~40% of the lateral membrane surface at any time, can control cellular scale patterning. Ankyrin-G microdomains require DHHC5/8 palmitoyltransferases which mediate the palmitoylation of ankyrin-G necessary for its membrane association (1). β II-spectrin also is recruited to these domains both through interaction with ankyrin-G as well as through binding to plasma membrane phosphoinositide lipids (1). Here, using live imaging of MDCK cells, we demonstrate that ankyrin-G microdomains move within the plane of the lateral membrane in a microtubule-dependent manner and sample almost the entire lateral membrane surface within 30 minutes. We propose that these ankyrin-G/ β II-spectrin microdomains promote lateral membrane biogenesis through inhibiting bulk endocytosis from the lateral membrane. The locally dynamic ankyrin-G/ β II-spectrin microdomains likely act by preventing endocytic pit formation, which occurs on the order of 1-5 minutes in the case of clathrin. We found that loss of lateral membrane height caused by depletion of either ankyrin-G or β II-spectrin can be reversed by pharmacological inhibition of endocytosis or through shRNA-mediated silencing of clathrin heavy chain. In parallel studies in neurons, we have also found that a giant isoform of ankyrin-G, but not its C70 mutant lacking a palmitoylation site, localizes to extrasynaptic plasma membrane microdomains in pyramidal neurons and stabilizes GABAergic synapses on the somatodendritic membrane through interactions with the GABA receptor-associated protein GABARAP. Inhibition of endocytosis restores GABAergic synapses that are lost in the absence of giant ankyrin-G. These results suggest that inhibition of endocytosis by locally dynamic ankyrin-G microdomains dependent on palmitoylation, phosphoinositides and microtubule-based movement may be a general mechanism for higher order cellular scale patterning.

1. He M, Abdi KM, & Bennett V (2014) Ankyrin-G palmitoylation and β II-spectrin binding to phosphoinositide lipids drive lateral membrane assembly. *J Cell Biol* 206(2):273-288.

P1329**Board Number: B673****The essential yeast type IV P-type ATPase Neo1 plays a role in establishing phosphatidylethanolamine asymmetry of the plasma membrane.**M. Takar¹;¹Biological Sciences, Vanderbilt University, Nashville, TN

Phospholipid asymmetry is a key feature of the eukaryotic plasma membrane (PM) that is required for the proper functioning of integral and peripheral membrane proteins. Type IV P-type ATPases (P4-ATPases) establish phospholipid asymmetry across the PM by pumping aminophospholipids, such as phosphatidylserine (PS) and phosphatidylethanolamine (PE), from the exofacial leaflet to the cytosolic leaflet. The P4-ATPases are highly conserved among eukaryotes and loss of P4-ATPase function is implicated in pathological conditions such as type II diabetes, obesity, intrahepatic cholestasis, and male infertility. P4-ATPases also play crucial roles in vesicle-mediated trafficking from Golgi and endosomal membranes. Although a canonical substrate transport pathway is well defined for some ion-transporting P-type ATPases, it is still under investigation how P4-ATPases translocate their bulky and amphipathic phospholipid substrates across the hydrophobic environment of the membrane. Previous work by the Graham lab suggests that a non-canonical transport pathway is used for phospholipid recognition and translocation by the yeast P4-ATPases Drs2 and Dnf1, which translocate PS and lyso-phospholipids (phospholipids with one acyl chain), respectively. However, it is not clear if all P4-ATPases use the same pathway and it remains to be determined what the phospholipid substrates are for all 5 yeast P4-ATPases (Dnf1, Dnf2, Dnf3, Drs2, and Neo1). In the current study, we investigated Neo1, an essential yeast P4-ATPase with unknown substrate specificity, which has been previously implicated in COPI retrograde trafficking. Neo1 localizes to Golgi membranes and inactivation of Neo1 activity leads to aberrant exposure of PE at the PM, suggesting a loss of PE flippase activity. Furthermore, an ATPase-dead Neo1 mutant, which can no longer flip phospholipids, cannot suppress this PE asymmetry defect. This finding suggests that the PE asymmetry defect is specifically due to a loss of Neo1 flippase activity. Interestingly, overexpression of Neo1 partially suppresses the membrane asymmetry defects associated with loss of Drs2 flippase activity (PE and PS are exposed in exofacial leaflet), but does not suppress the asymmetry defects when Dnf1 and Dnf2 flippase activity is inactivated (lyso-PS and lyso-PE are exposed). In addition, overexpression of wild-type Drs2 does not suppress Neo1 deficiency, but a Drs2-[QQ→GA] mutant defective for PS recognition but with enhanced ability to flip PE effectively suppresses Neo1 deficiency. Based on these findings, we hypothesize that the essential function of Neo1 is to flip diacylated PE across the Golgi membrane to the cytosolic leaflet.

P1330**Board Number: B674****INVESTIGATING THE ROLE OF THE PHOSPHOLIPID CARDIOLIPIN IN THE REGULATION OF MITOCHONDRIAL BIOENERGETICS.**M. Sathappa¹, M. Greenwood¹, M. Baile², S.M. Claypool², N. Alder¹;¹Molecular and Cell Biology, University of Connecticut, Storrs, CT, ²Johns Hopkins University School of Medicine, Baltimore, MD

Lipids are known to be critical determinants of membrane protein topology, conformation, and function. Cardiolipin (CL) is involved in the assembly and operation of mitochondrial respiratory complexes. However, attempts to elucidate the molecular mechanisms by which CL activates OXPHOS complexes have been confounded by technical limitations. On the one hand, analysis of individual respiratory complex activity and redox potentials in intact mitochondria does not permit the evaluation of specific lipid composition and is hindered by the presence of other coupled complexes. On the other hand, individual complexes can be isolated in detergent micelles, but this leads to delipidation of annular lipids, misfolding and inactivation. The goal of this study is to analyze which physiochemical properties of CL are important in activating respiratory enzymes, using Cytochrome C Oxidase (COX) as a model. To this end, we have reconstituted the purified COX complex into soluble, monodisperse, and stable nanodiscs of precisely defined lipid composition with a range of CL variants. Specifically, we have addressed which properties of the CL head group and acyl chain regions are important for the enzymatic activity and heme coordination of this enzyme.

P1331**Board Number: B675****Role of ER-Golgi contact sites in sphingolipid synthesis in yeast.**L. LIU¹;¹NIDDK, NIH, BETHESDA, MD

Membrane contact sites are regions where two organelles come in close proximity and are thought to be sites where lipids and signals are exchanged between organelles. We wanted to investigate the role of close contacts between the ER and Golgi complex in nonvesicular lipid transfer. Ceramides are synthesized in the ER and are delivered to the Golgi by vesicular and non-vesicular pathways, where they are converted to sphingolipids. It has previously been shown that in yeast ceramide transfer from the ER to Golgi is partially blocked in mutants with defects in vesicular transfer such as *sec18-1ts* cells (which have a conditional defect in the yeast homolog of NSF) or in cells missing the oxysterol-binding proteins Osh2p, Osh3p, and Osh4p. The ER to Golgi ceramide transfer that is not blocked in these strains is thought to be nonvesicular and may occur at ER-Golgi contact sites. To better understand this nonvesicular transport, we engineered several artificial ER-Golgi tethers. Remarkably, these tethers significantly increased nonvesicular ceramide transfer in *sec18-1ts* cells and in *osh2-4Δ* cells, both in vivo and in vitro, suggesting that tethering of the ER and Golgi facilitates ceramide transfer. To identify

other proteins that facilitate ceramide transfer, we made use of the fact that *osh2-4Δ* cells are hypersensitive to aureobasidin A (AbA), an inhibitor of sphingolipid biosynthesis. We screened for proteins that when overexpressed allow *osh2-4Δ* cells to grow in the presence of AbA. Some of the proteins we identified may tether the ER and Golgi or facilitate ceramide transfer between these organelles. Characterization of these proteins will reveal more about the mechanism and regulation of nonvesicular ceramide transfer between the ER and Golgi complex.

P1332

Board Number: B676

Nir2 plays a central role in ER-PM junctions maintaining Phosphoinositide Signaling Competence.

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Nir2 plays a central role in ER-PM junctions maintaining Phosphoinositide Signaling Competence

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During phospholipase C activation, large amounts of phosphoinositide lipids are consumed while generating the second messengers, diacylglycerol (DG) and InsP₃. The small PtdIns(4,5)P₂ pool is turned over multiple times and it has been evident that the plasma membrane (PM) inositide pools need to be replenished for sustained signaling with newly synthesized phosphatidylinositol (PtdIns) originating from the ER. Conversely, phosphatidic acid (PtdOH) generated via DG in the PM has to return to the ER where it is utilized for PtdIns synthesis. Although PtdIns transfer protein has been postulated to transport PtdIns from the ER to the PM, the protein responsible for PtdOH transport in the other direction has remained elusive. In this study we used a combination of methods all based on intact cells and found that depletion of Nir2, a homolog of the *Drosophila* RdgB protein, which has previously been identified as a PtdIns transfer protein, causes a defect both in the utilization of PtdOH at the PM and the synthesis of PtdIns in the ER during PLC activation. Conversely, overexpression of Nir2 facilitates PtdOH removal from the PM and its conversion to PtdIns in agonist-stimulated cells. These data together with the agonist-induced translocation of Nir2 into ER-PM contact sites suggest that Nir2 plays a critical role in the recycling of the lipid products of PLC activation and that the agonist-induced increased turnover of PtdIns described several decades ago is spatially confined to ER-PM contact zones.

P1333**Board Number: B677****Alterations in caveolar morphology produced by varying plasmalemmal cholesterol, revealed by quick-freeze, deep-etch electron microscopy.**A. Yoshimura¹, J.E. Heuser²;¹Institute for Integrated Cell-Material Sciences, Kyoto University, Kyoto, Japan, ²iCeMS, Kyoto University, Kyoto, Japan

The 'quick-freeze, deep-etch' technique of EM provides high-resolution, 3-D topology of biological structures and is ideal for studying the plasma membrane (PM) surface and associated structures. When applied to "unroofed" cells, it displays the striated coats on caveolae with unprecedented clarity. Although it has long been evident that caveolae display different degrees of curvature, the reason for this has never been understood. To begin to answer this, we here sought to determine whether caveolae are capable of changing their curvature, and capable of doing so reversibly. Using deep-etch EM, which allowed us to detect all the caveolae on the PM, we examined the effect of acute manipulations of PM cholesterol-levels on their curvature. For this study, we chose a suitable cell line with a simple morphology but with plenty of caveolae (T24 cells, originally from a human bladder cancer).

At normal 'control' cholesterol levels, T24 cells display caveolae with a whole range of curvatures, from completely flat to highly invaginated, all of which display the characteristic striated coats. Decreasing PM cholesterol levels by applying methyl-beta-cyclodextrin induces dramatic flattening of the majority of caveolae, as has long been known, but their striated coats remain unchanged. On the other hand, elevating PM cholesterol with cholesterol-charged cyclodextrin induces increase in caveolar curvature, and also causes a complete dissolution of their coats into unusually thin and dispersed 'stripes' that reveal for the first time the basic underlying organization of the caveolin proteins in their coats.

Importantly, these structural changes can be induced either by applying the agents that elevate or lower PM cholesterol to living cells and *then* unroofing them, or by applying the same agents to isolated PM sheets or 'lawns' made from cells unroofed *prior* to treatment. This demonstrates that the observed changes in caveolar morphology are purely biophysical phenomena that do not require any cellular metabolism or activity.

Additionally, we could show that these changes are *reversible*, since when PM cholesterol is first lowered in order to flatten the majority of caveolae, and then is re-elevated again, caveolae still swell and invaginate and their coats still disperse; and again, this works even if the re-elevation of cholesterol is not done until *after* the unroofing. These results demonstrate that caveolae are capable of re-invaginating from the flat state without any cellular activities, and that they respond *directly* to PM cholesterol-levels and the physical state of the bilayer, by majorly altering their curvature.

P1334**Board Number: B678****Reinforcement of organelle identity by the ERAD ubiquitin ligase Doa10.**A. Ruggiano¹, P. Carvalho²;¹Cell and Developmental Biology, Centre de Regulacio Genomica , Barcelona, Spain, ²Cell and Developmental Biology Programme, Center for Genomic Regulation (CRG), Barcelona, Spain

ER-associated protein degradation (ERAD) is a quality control pathway for clearance of misfolded proteins from the endoplasmic reticulum (ER) through the ubiquitin-proteasome system. Beyond this well-studied role, ERAD controls the levels of some functional but short-lived ER proteins by targeting them for degradation under a specific condition, thereby in a regulated fashion. Despite its importance in ER homeostasis, regulated degradation through ERAD accounts for only few examples. We identified novel endogenous ERAD substrates in the yeast *S.cerevisiae* by using quantitative proteomics. Among these were some lipid droplets (LDs) proteins. LDs are organelles originating from the ER, where LD-specific proteins are synthesized before being targeted to their surface. We show that some LD membrane proteins are degraded by the ERAD ubiquitin ligase Doa10 when localizing in the ER, and that the membrane anchor is necessary for this process. Accumulation of LD proteins in the ER leads to a growth defect, indicating that their degradation is important for maintaining organelle homeostasis. LD proteins misplaced in the ER represent a novel class of ERAD substrates and uncover a role for this pathway in reinforcement of organelle identity.

P1335**Board Number: B679****Very Long Acyl Chain Sphingolipids Prefer Tail-to-Tail interaction with Cholesterol, which Restricts Microdomain Formation.**K.C. Courtney¹, C. Zhang², X. Zha^{1,2};¹Department of Biochemistry, Microbiology Immunology, University of Ottawa, Ottawa, ON, ²Chronic Disease Program, Ottawa Hospital Research Institute, Ottawa, ON

Affiliation between cholesterol and sphingolipids is essential for the formation of lateral microdomains or lipid rafts in both model membranes and isolated plasma membranes. However, such microdomains are not observed in live cells and, particularly, little is known how cholesterol interacts with very long acyl chain sphingolipids (22:0-24:0), the most abundant species of sphingolipids in mammalian cell membranes. Here we report that cholesterol preferred a novel tail-to-tail interdigitation with 24:0 sphingomyelin (SM), contrast to its well-known side-by-side affiliation with 16:0 or 18:0 SM. In live cell plasma membrane where very long acyl chain sphingolipids are exclusively in the exofacial leaflet, such interdigitation restricted the majority of cholesterol to the cytoplasmic leaflet. Consequently, giant unilamellar vesicles (GUVs) with very long acyl chain SM in the outer leaflet do not form visible microdomains, although the ones with 16:0 SM do. Thus, interdigitation is the primary mode of

interaction between cholesterol and sphingolipids in mammalian cell membranes, thereby restricting microdomain formation.

Mitochondria 1

P1336

Board Number: B680

Exploring semithin sections by atomic force microscopy to visualize cell structure.

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Cell structure has been abundantly documented by light and electron microscopy. Recently visualization of by atomic force microscopy has been used as to explore different cell components at the nanoscale. Using an approach including the processing of samples as for transmission electron microscopy we have been able to visualize and recognize several different organelles and cell structures. We prepared different samples including plants as *Lacandonia schismatica*, *Allium cepa*, *Ginkgo biloba* and *Araucaria heterophylla* sp., animal cells as Hep" or HeLa cells lines or rat hepatocytes and thymus, as well as trophozoites of *Entamoeba histolytica* for standard transmission electron microscopy and analyzed stained or unstained semithin sections placed onto glass slides. Semithin sections about 250 nm width were obtained using a glass or diamond knife. They were then probed by atomic force microscopy operating either in contact or intermittent mode. Cell nuclei and nucleoli, nuclear bodies and particles and pores have been observed. Moreover, compact chromatin has been also recognized. In addition, chloroplasts and mitochondria were visualized and recognized among other cell structures. These observations allow us to consider using atomic force microscopy to study different cell structures and processes at the nanoscale (DGAPA-UNAM PAPIIT IN220713; PAPIIME PE211412).

P1337**Board Number: B681****Site-specific reactivity of non-enzymatic lysine acetylation.**J. Baeza^{1,2}, J.M. Denu^{1,2}, M.J. Smallegan²;¹Biomolecular Chemistry, Univ Wisconsin-Madison, Madison, WI, ²Epigenetics, Wisconsin Institute for Discovery, Madison, WI

Protein lysine acetylation is now considered a major regulatory modification affecting diverse cellular processes. Unlike nuclear acetylation, which is catalyzed by several lysine acetyltransferases (KATs) families, direct evidence for a mitochondrial acetyltransferase is lacking, raising the possibility that acetylation may be uncatalyzed whereby the lysine side chain reacts with the thioester of acetyl-CoA. Due to the higher pH and acetyl-CoA levels in mitochondria, non-enzymatic acetylation appears to be a viable mechanism. Consistent with this idea, *in vivo* studies in which acetyl-CoA levels in mitochondria are altered results in increased acetylation. Furthermore, *in vitro* incubation of protein with acetyl-CoA also leads to a time dependent increase in acetylation.

In order to establish the feasibility of non-enzymatic acetylation, the chemical kinetics of reactivity must be quantified. Here, we performed a kinetic study to determine the rate of reactivities of site-specific, non-enzymatic acetylation of purified mitochondrial and non-mitochondrial proteins using acetyl-CoA and acetyl-phosphate. By utilizing a newly developed mass spectrometry method, we determined the rates for 98 lysine sites, quantifying reactivities ranging over three orders of magnitude. We measured the highest reactivity for glutamate dehydrogenase lysine 503 with a 2nd order rate constant of 2.73 h⁻¹ mM⁻¹ (R² 0.9874). Together, this study indicates that non-enzymatic lysine acetylation is a viable mechanism and has implications on how we view regulatory, post-translational modifications.

P1338**Board Number: B682****Mitochondrial maintenance changes dynamically with age in *Caenorhabditis elegans* adult neurons.**N. Morsci¹, D.H. Hall², M. Driscoll³, Z. Sheng¹;¹National Institutes of Health, NINDS, Bethesda, MD, ²Neuroscience, Albert Einstein College of Medicine, Bronx, NY, ³Molecular Biology and Biochemistry, Rutgers University, Piscataway, NJ

Aging is associated with cognitive decline and increasing risk of neurodegeneration. Mitochondrial dysfunction has been implicated in etiology of neurodegeneration, raising a fundamental question: Does organismal aging affect mitochondrial maintenance in neurons? By *in vivo* analysis of mitochondria in individual *Caenorhabditis elegans* neurons, we performed a comprehensive analysis of age-associated changes in mitochondria morphology, density, trafficking, and stress resistance throughout adult life. Adult neurons display three distinct stages of increase, maintenance and decrease in mitochondrial size and density throughout adulthood. Mitochondrial trafficking in the distal mechanosensory process declines progressively with age starting from early adulthood. In contrast, long-lived *daf-2* mutants

exhibit delayed age-associated changes in mitochondrial morphology, constant mitochondrial density, and maintained trafficking rates during adulthood. Finally, reduced mitochondrial load at late adulthood correlates with decreased mitochondrial resistance to oxidative stress. Our study provides the first *in vivo* evidence of dynamic changes in neuronal mitochondrial maintenance during organismal aging.

This work is supported by the Intramural Research Program of the National Institute of Neurological Disorders and Stroke, National Institutes of Health.

P1339

Board Number: B683

Mechanism Removing Damaged Mitochondria from Axonal Terminals.

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Mitochondrial dysfunction, altered dynamics, and impaired transport emerge as central problems associate with major neurodegenerative disorders. Sequestration of damaged mitochondria into autophagosomes and subsequent degradation within mature lysosomes constitute a key cellular pathway in mitochondrial quality control. Mature lysosomes are relatively enriched in the soma. Thus, an important mechanistic question, critical toward understanding axonal degeneration emerges: How are damaged mitochondria at distal synapses efficiently eliminated? By chronically dissipating mitochondrial membrane potential ($\Delta\psi_m$) in mature cortical neurons, we previously demonstrated that Parkin-mediated mitophagy mainly occurs in the soma and proximal regions of processes (Cai et al., *Current Biology* 2012). Under these chronic stress conditions, the majority of neurons survive and a significant portion of mitochondria remains motile, thus better reflecting chronic mitochondrial stress *in vivo* under pathophysiological conditions. In the current study, we detected a significant decrease of axonal mitochondrial docking protein syntaphilin following chronic $\Delta\psi_m$ dissipation. Mitochondrial retrograde transport is increased after mitochondrial depolarization for 6 hr. This phenotype is correlated with reduced levels of syntaphilin. Using the syntaphilin KO mouse model, we further confirmed that degradation of syntaphilin is essential for the depolarization-induced retrograde transport. Immunostaining analysis revealed that syntaphilin-containing vesicles are derived from depolarized mitochondria, some of which target to LAMP1-labeled late endosomal/lysosomal organelles. Our study demonstrates that syntaphilin is highly regulated by sensing $\Delta\psi_m$. Degradation of syntaphilin is required for enhanced retrograde transport, thus preferring damaged mitochondria trafficking from distal axons to the soma where major lysosome are mainly localized. Altogether, our study provides new mechanistic insight into the fine coordination of mitochondrial motility and the quality control in neurons. (Supported by the Intramural Research Program of NINDS, NIH)

P1340**Board Number: B684****The large subunit of Rubisco functions in oxidative stress tolerance and in the control of RNA oxidation in the chloroplast of the green alga *Chlamydomonas reinhardtii*.**J. Dhaliwal¹, Y. Zhan¹, J. Uniacke², W. Zerges¹;¹Biology, Concordia University, Montreal, QC, ²Molecular and Cellular Biology, University of Guelph, Guelph, ON

The large subunit of the Calvin cycle enzyme, Rubisco (RBCL), binds to RNA and localizes to stress granule-like bodies under oxidizing conditions in the chloroplast of the green alga *Chlamydomonas reinhardtii*. This suggests that RBCL has a “moonlighting” function during oxidative stress in the chloroplast. Through analysis of Rubisco mutants, we reveal here that RBCL controls the level of RNA oxidation and determines cell survival and viability during hydrogen peroxide-induced oxidative stress. Cellular subfractionation experiments demonstrate that a minor pool of RBCL carries out this moonlighting function independently of the Rubisco holoenzyme complex. Moreover, immunofluorescence microscopy reveals that oxidized RNA is localized in the pyrenoid of the chloroplast, the major intracellular location of RBCL. Together these results reveal a novel factor involved in RNA quality control in chloroplasts which functions in oxidative stress tolerance.

P1341**Board Number: B685****Preferential Distribution of Intermembrane Space and Matrix Proteins May Contribute to Mitochondrial Function.**K.S. Nadhan¹, S.P. Alla¹, N.E. Hoffman¹, M. Muniswamy¹;¹Center for Translational Medicine, Temple University School of Medicine, Philadelphia, PA

Background: The proton motive force (pmf) in the mitochondrion fuels many metabolic processes in the body. Current theory states that one major component of the pmf is the electrostatic force, created by proton flux across the inner membrane as electrons move down the ETC. However, it is possible that non-permeable particles within the matrix and intermembrane space (IMS) contribute to the large electrostatic gradient and pH that is used to generate the pmf. As of now, the role played by the IMS and matrix proteins in the generation and maintenance of pmf is unclear. Consequently, we studied different characteristics of the proteomes in the matrix and IMS to see if they could potentially play a role.

Methods: The Human MitoCarta Database was used to make a library of proteins for both the matrix and IMS. We analyzed primary protein structures to determine the number of acidic and basic residues found in each amino acid sequence, and subsequently calculated their relative frequencies within each

protein. Then using a Scripps Institute software, we calculated the pI, mw, and charge density for each individual protein.

Results: Data analysis showed a statistically significant difference ($p < 0.001$; $n=39$, $n=114$) between the percentage of charged amino acids that exist in IMS (30.6%) and matrix proteins (26.8%). No significance was noted among the relative frequencies of basic residues within two groups of proteins. However, we found a significant difference ($p < 0.001$) between the percentage of acidic amino acid residues, glutamic acid (glu) and aspartic acid (asp), in the proteins of the IMS (14.7%) and the matrix (11.4%). IMS proteins have, on average, a 23.2% greater proportion of asp residues in their primary amino acid sequence ($p < 0.001$) and also an average a 34.5% greater proportion of glu residues ($p < 0.001$). Moreover, results show that matrix proteins have larger pI values ($p < 0.001$) and higher mw distribution ($p < 0.002$) than IMS proteins. Then by using the pI and the mw data, we compared the charge densities between the two compartments and found a statistical difference ($p = 0.001$).

Conclusion: Our data shows an intrinsic difference in the production- on a genetic, transcriptional, or translational level- of the proteins destined for either compartment. There is a larger proportion of acidic amino acids in IMS proteins than matrix proteins, contributing a larger negative charge to the IMS. This observed difference in charge supports the hypothesis that proteins within the mitochondrial compartments contribute to the electrostatic gradient required to produce ATP in the mitochondria. The observation that the proteins in the IMS have lower mw with larger value charge densities does not clearly contribute to our hypothesis and needs further exploration.

P1342

Board Number: B686

Yeast mitochondria as a model system to decipher the biogenesis of bacterial outer membrane proteins.

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The vast majority of outer-membrane proteins in Gram-negative bacteria belongs to the class of membrane-embedded β -barrel proteins. In addition to Gram-negative bacteria, the presence of β -barrel proteins is restricted to the outer membranes of the eukaryotic organelles mitochondria and chloroplasts, which according to the endosymbiotic theory arose from the engulfment of a prokaryote by the ancestral eukaryotic cell. The assembly of these proteins into the corresponding outer membrane is facilitated in each case by a dedicated protein complex that contains a highly conserved central β -barrel protein termed BamA/YaeT/Omp85 in Gram-negative bacteria, Tob55/Sam50 in mitochondria, and Toc75/Oep80 in chloroplasts. To date, little is known about how the aforementioned complexes allow for the integration of β -barrel precursors into the lipid bilayer. Interestingly, previous studies showed that during evolution, these complexes retained the ability to functionally assemble β -barrel proteins from different origins. Taking advantage of the evolutionary conservation in the biogenesis of β -barrel proteins we use yeast mitochondria as a model system to decipher the biogenesis of the

trimeric autotransporter protein Yersinia adhesin A (YadA). Upon expression in yeast cells YadA was assembled into the mitochondrial outer membrane in its native conformation in a process dependent on the TOM and TOB complexes. Interestingly, the mitochondrial targeting of the bacterial periplasmic chaperone Skp resulted in elevated levels of YadA. A similar effect could not be observed for the chaperones SurA or SecB. Taken together, these results demonstrate that during evolution, mitochondria preserved the ability to functionally assemble prokaryotic proteins belonging to a class that cannot be found in any eukaryotic cell.

Currently, we start to use this model system to decipher the biogenesis and assembly of bacterial secretins belonging to the type II, type III and type IV secretion systems.

P1343

Board Number: B687

Identification of mitochondria-associated mRNA through tight binding to Tom20 and Puf3.

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The localization of mRNAs to specific regions of the cell contributes to proper post-translational protein localization and function. Mechanisms that regulate the inheritance of proper types of maternal mRNA in the daughter cells are not yet well understood. The idea that organelles act as scaffolds for the localization of mRNAs has been tested. Some mRNAs expressed during S phase were localized actively to daughters by hitchhiking on the cortical endoplasmic reticulum (cER). Mitochondria are vital and well-characterized compartments in the cell and are inherited by the daughter cells. It is already well established that many nuclear-encoded mRNAs are localized to the outer membrane of the mitochondria (ML-mRNA) in a Tom20 and Puf3-dependent manner by microarray analysis of biochemical mitochondrial fractions. However, the relation between mitochondria inheritance and RNA inheritance is still in question. Tom20, a mitochondria transporter component, recognizes the MTS peptide coming out of the ribosome, then anchors the translating complex (mRNA, ribosome and nascent peptide) on the mitochondrial outer membrane. The Pumilio family RNA binding protein Puf3 binds to the ORF and the 3'-UTR of specific mRNAs to localize them to the mitochondria outer membrane independent of translation.

To determine how ML-mRNA localization is regulated by mitochondrial inheritance and how this affects intracellular function, we first performed an RNA-seq based screen for mRNAs that are associated with mitochondria through tight binding to GFP-tagged Tom20 and Puf3. Our results showed that GFP-tagged Tom20 and Puf3 are localized to mitochondria as expected. We verified that these tagged proteins fully complement *tom20Δ* and *puf3Δ* mutants in terms not only of the cell viability but also of mitochondrial health. The over-expression of Tom20 caused swollen, misshapen mitochondria. Therefore, the effect of expressing GFP-tagged Tom20 on mitochondrial morphology was evaluated to ensure that mRNA was purified only from healthy mitochondria with normal mitochondrial morphology. We confirmed that

several specific mRNAs (*FUM1*, *ATP2*, *OXA1*, *BCS1*) bind to Tom20 in a translation-dependent manner and to Puf3 in a translation-independent manner by preparing samples with and without Cycloheximide or EDTA. We are performing data analysis of RNA-seq to identify novel ML-mRNAs. We will visualize these mRNAs and quantify the changes in their localization during mitochondrial inheritance by using live-cell quantitative 3D microscopy. In doing so, this research will provide insights into how the molecular components surrounding mitochondria are influenced by mitochondria inheritance.

P1344

Board Number: B688

Actin as an integrator of mitochondrial behavior.

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In order to integrate with the intrinsic and environmental cues, mitochondrial networks in living cells are constantly changing. A balance between fusion and division rates is necessary for the maintenance of this network, but it is not sufficient: motility and tethering are additional activities needed for maintenance of correct mitochondrial morphology. Components that regulate these processes are known, but it is less understood how these activities are integrated at a systems level. Actin is a potential candidate integrator as it is a known polarity sensor and is involved in mitochondrial motility, positioning and division in many cell types. Using the relatively simple and well-characterized mitochondrial network system of budding yeast, we tested this idea by altering the actin cytoskeleton and examining mitochondria in wild type and mutant strains disrupted in activities that play a direct role in the maintenance of mitochondrial shape and distribution. Consistent with previously published work, treatment of wild type yeast cells with 100 μ M Latrunculin A, a concentration that causes complete depolymerization of F-actin in cells, caused massive fragmentation of the mitochondrial network, indicating that F-actin plays a critical role in maintenance of mitochondrial structure. In contrast, treatment of yeast cells with 10 μ M Latrunculin A, a concentration previously shown to preferentially disrupt actin cables and not actin patches, caused mitochondrial networks to transform into net-like structures - a phenotype similar to division mutants (e.g. Δ dnm1 cells), suggesting a role(s) for actin in mitochondrial division. We examined the effects of 10 μ M Latrunculin A on mitochondria in Δ dnm1 cells and did not observe an additive effect on mitochondrial net formation. However, we observed that cortically-localized mitochondrial nets in Δ dnm1 cells significantly expanded, as previously described. This phenotype suggests the possibility that F-actin could also play a role in tethering the organelle at the cortex. Accordingly, we examined the effect of 10 μ M Latrunculin A on mitochondria in cells lacking Num1, a key component of a mother-specific mitochondrial cortical tether. In Δ num1 cells, in the absence of Latrunculin A treatment, we observed that a population of mitochondria were still persistently tethered at the mother cortex, indicating the presence of a distinct Num1-independent cortical tether. Significantly, treatment of Δ num1 cells with 10 μ M Latrunculin A abolished the

association of mitochondria with the cortex, suggesting that the Num1-independent tether is actin-dependent. Together with published observations, our data indicate that actin is involved in mitochondrial division, tethering and motility. Therefore, we propose that actin functions as an integrator of the various processes required for maintenance of mitochondrial networks in budding yeast.

P1345

Board Number: B689

Neuronal Mitochondria changes morphology through the activities of phosphatases and kinases in *Caenorhabditis elegans*.

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Mitochondria are dynamic organelles that changes their shape and size via fission and fusion. Mitochondria is divided by Drp1 and Fis1, whereas mitochondria is fused by Fzo1, OPA1, and Mgm1 in mammalian cells. DRP-1 and EAT-3 of *Caenorhabditis elegans* are required for fission and fusion, respectively. Calcineurin and several kinases regulate the activity of Drp1. Dephosphorylation of Drp1 by calcineurin promote mitochondrial fission. Also, by regulating phosphorylation of Drp1, PKA inactivate mitochondrial fission, whereas Cdk1 and CaMK α activate mitochondrial fission. In this research, we studied about mitochondrial dynamics in neurons affected by phosphatases and kinases in *C. elegans*, using feeding RNAi approach. This research will provide the insight about mitochondrial dynamics in touch sensory neurons and help understand mechanosensory behavior in *C. elegans*.

P1346

Board Number: B690

The topology of Sequestosome 1/p62 in mitochondria and its effects on mitochondrial morphology.

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Sequestosome 1/p62 is a highly conserved, ubiquitin-binding protein that is present within inclusions associated with many neurodegenerative diseases. Mitochondrial dysfunction is a major characteristic of neurodegenerative diseases. Growing lines of evidence correlates p62 with damaged mitochondria under stress conditions. However, the specific location and functions of p62 in mitochondria are poorly understood. We found p62 is localized in mitochondria under physiological conditions and defects of p62 lead to mitochondrial morphology change and dysfunctions. We hypothesized that the orientation in which p62 localized in mitochondria affects its function. The aim of this project is to investigate the topology of p62 in mitochondria and what roles it might play. Results of various truncated p62 constructs distribution in cells suggested that N-terminal aa62-115 is crucial for p62 mitochondria

localization. Tryptic digestion and protein solubility experiments indicated that p62 is localized to mitochondria with N-terminus associating with the mitochondrial inner membrane and C-terminus associating with the outer membrane. We also found that p62 might regulate dynamic interactions between the mitochondrial outer membrane and inner membrane sensed by the cell fission/fusion machinery. Immunofluorescence staining showed that the N-terminal p62 construct (1-266) recovered mitochondrial morphology of p62 KO MEF cells, but the C-terminal construct of p62 (230-440) did not. This result might be caused by the interaction between p62 and mitochondrial membrane receptor proteins relating to protein import. Further investigation is undergoing to clarify the mechanism. This study illustrated the topology of p62 in mitochondria and provided preliminary data to define the functions of p62 in mitochondria involved in neurodegenerative diseases.

P1347

Board Number: B691

Transient assembly of F-actin on the outer mitochondrial membrane contributes to mitochondrial fission.

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Mitochondrial dynamics, including fission and fusion, are vital for supplying cellular energy as well as controlling other tasks including apoptosis, aging and cellular differentiation. Defective mitochondrial fission and fusion have been implicated in a wide spectrum of human diseases including Parkinson's disease, Alzheimer's disease and many others. Although recent findings point to a role of actin cytoskeleton in regulating mitochondrial fission, little is known about the molecular mechanism of how actin controls this process. Here, we report that transient *de novo* polymerization of F-actin on the outer mitochondrial membrane (OMM) contributes to Drp1-dependent mitochondrial fission in mammalian cells.

We found that in cells treated with mitochondrial inducing triggers, F-actin transiently accumulated on OMM prior to Drp1-mediated steps of this process. Furthermore, mitochondrial accumulation of F-actin was also detected in mitotic cells, in which mitochondrial fragmentation contributes to stochastic distribution of these organelles into daughter cells. Thus, mitochondrial assembly of F-actin occurs in both, stress-induced and physiological Drp1-dependent mitochondria fission.

Furthermore, pretreatment with Latrunculin B, a potent actin polymerization inhibitor, completely hindered the mitochondrial accumulation of F-actin in fission inducers-treated cells, indicating that *de novo* actin polymerization is required for mitochondrial assembly of F-actin. In addition, mitochondrial accumulation of F-actin was prolonged in Drp1^{-/-} MEFs and Mff^{-/-} MEFs, but not in Mfn2^{-/-} MEFs suggesting that inhibition of mitochondrial fission by loss of functional Drp1 also hinders disassembly of F-actin on the mitochondria. These results support the possibility that mitochondrial assembly of F-actin is a step in Drp1-dependent mitochondrial fission.

To identify actin-modifying proteins that are involved in this process, we performed an shRNA screen of various actin regulatory proteins. The data showed that downregulation of p34 and Arp2, two factors of Arp2/3 F-actin nucleation complex, as well as cortactin or cofilin, resulted in mitochondrial elongation, a phenotype consistent with the inhibition of mitochondrial fission. Consistent with the role of these proteins in the mitochondrial assembly of F-actin, quantitative colocalization analysis revealed significant increases in Arp2/3 complex, cortactin and cofilin association with mitochondria in Drp1^{-/-} cells, supporting a role for actin-modifying proteins in the mitochondrial assembly of F-actin. Together these findings shed light on the increasing importance of actin in the regulation of mitochondrial fission

P1348

Board Number: B692

The Effects of Pioglitazone on Liver Cell Bioenergetics.

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Insulin resistance is linked to mitochondrial dysfunction and decreased oxidative capacity of metabolically active tissues. Pioglitazone is commonly used to treat type-II diabetes and was shown to interact with the small iron-cluster containing protein mitoNEET (mNT, CISD1), which localizes to the mitochondrion. We recently observed that human hepatocellular carcinoma cells (HepG2) cultured in presence of the glucose epimer galactose (10 mM) at low glucose concentrations (<1 mM) express significantly reduced levels of mNT compared to cells cultured in presence of 15 mM glucose alone. We hypothesized that pioglitazone treatment may alter cellular bioenergetics through interaction with mNT and employed respirometry and calorimetry to investigate the effects of pioglitazone treatment on HepG2 cells cultured in high glucose medium or low glucose plus galactose medium. After 24 h of pioglitazone treatment with concentrations ranging from 15 μ M to 60 μ M, only cells cultured in the galactose medium showed a reduction in cell proliferation rates. However, cells cultured in either medium showed significant decreases in proliferation rates at all pioglitazone concentrations investigated after 48 h of exposure. Respiration rates of permeabilized cells in the presence of mitochondrial substrates and ADP (OXPHOS) were significantly increased for HepG2 cells cultured in galactose ($45.8 \pm 1.96 \text{ pmol O}_2 \cdot \text{s}^{-1} \cdot 10^{-6} \text{ cells}$; $n = 6, \pm \text{SE}$) compared to cells cultured in 15 mM glucose alone ($30.5 \pm 2.94 \text{ pmol O}_2 \cdot \text{s}^{-1} \cdot 10^{-6} \text{ cells}$; $n = 6, \pm \text{SE}$). Acute exposure to 60 μ M pioglitazone caused a severe reduction in OXPHOS for cells cultured in either medium (galactose: $12.18 \pm 1.37 \text{ pmol O}_2 \cdot \text{s}^{-1} \cdot 10^{-6} \text{ cells}$; glucose: $10.1 \pm 1.01 \text{ pmol O}_2 \cdot \text{s}^{-1} \cdot 10^{-6} \text{ cells}$). Overall heat flow of intact cells was $55.6 \text{ mW} \pm 3.0 \text{ mW}$ per million cells in glucose medium and was significantly reduced to $47.2 \text{ mW} \pm 2.5 \text{ mW}$ per million cells ($n = 10, \pm \text{SE}$) if cells were cultured in presence of galactose. Surprisingly, acute treatment of cells with 60 μ M pioglitazone increased heat dissipation in cells grown in glucose medium by $\sim 20\%$, but the effect was significantly lower for cells cultured in the presence of galactose. Our results demonstrate that pioglitazone targets multiple components of the mitochondrial OXPHOS machinery, but reductions

in cellular proliferation are ameliorated in high glucose medium through an increase in anaerobic metabolic activity. Further studies are needed to elucidate the role of mNT in the impact of pioglitazone on cellular energy transduction. (The EIU College of Sciences is gratefully acknowledged for a generous stipend)

P1349

Board Number: B693

Inhibition of PGC-1 α is responsible for Mitochondrial Defects in Hutchinson-Gilford Progeria Syndrome.

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Mitochondrial dysfunction, including a reduction of mitochondrial biogenesis and accumulation of mitochondrial DNA (mtDNA) mutations, has been postulated as a core player in the normal aging process. Hutchinson-Gilford progeria syndrome (HGPS), caused by a single nucleotide mutation in the lamin A gene (*LMNA*), is a rare and fatal premature aging disease. To investigate the potential contributions of mitochondrial dysfunction in HGPS pathogenesis, we conducted morphological and functional characterizations of mitochondria in primary HGPS fibroblasts. Super resolution microscopy analysis revealed an increase in swollen and fragmented mitochondria and a decrease in mitochondrial mobility in HGPS cells. Importantly, PGC-1a, a key transcriptional regulator of mitochondrial biogenesis, was significantly downregulated in HGPS fibroblasts, suggesting PGC-1a reduction may contribute to the mitochondrial defects in HGPS. Moreover, we found that the treatment with an antioxidant methylene blue alleviated senescence and mitochondrial phenotypes associated with HGPS fibroblasts and prolonged life span. Together, our study supports that the mitochondrial dysfunction contributes to the premature aging phenotypes in HGPS and suggests possible new directions to treat this devastating disease.

P1350

Board Number: B694

Dantrolene attenuates calcium release from mitochondria during potassium induced depolarization in *Paramecium*.

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Paramecium are excitable cells whose swimming behavior is regulated by membrane potential. Backward swimming responses can be initiated by depolarizing the cell with an increase in external K⁺ added to the bathing solution. We have identified a ryanodine-sensitive calcium flux in *Paramecium* that can be observed under such depolarizing conditions. When *Paramecium* were treated with ryanodine

receptor antagonists, backward swimming responses initiated by K^+ depolarization were significantly reduced, but not eliminated. Our attempts to localize these channels led not to the endoplasmic reticulum (the expected site of ryanodine receptor/channels) but to mitochondria. Increasing evidence suggests that ryanodine sensitive channels can be found in mitochondria. In addition, recent sequence analysis of the *Paramecium* genome reveals several channels with conserved ryanodine domains, although none with significant overall homology. Since Ca^{2+} is a mediator of the backward swimming response, we used the mitochondrial calcium-sensitive dye Rhod-2 to assess mitochondrial calcium contributions to depolarization. During a K^+ – induced depolarization, Rhod-2 fluorescence in the mitochondria decreased by approximately 30%. When cells were treated with the ryanodine receptor antagonist dantrolene prior to depolarization, the loss of calcium from mitochondria was significantly attenuated. We conclude that mitochondrial calcium stores extend backward swimming responses in *Paramecium*.

P1351

Board Number: B695

The diversity of Mitochondrial Intermembrane Space and Matrix proteomes may potentially play a key role in tissue energetics and metabolic processes.

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Background: The variety of tissues in our body have different energetic and metabolic needs. In order to appropriately meet these various levels of need, they constantly maintain the oxidative phosphorylation capacities of their mitochondria. This implies that the mitochondria themselves may also need to be fundamentally different; however, the source of this difference is unclear. One source of this possibility may be at the protein level. Hence, we studied the soluble or non-membrane proteomes present in the matrix (M) and intermembrane space (IMS) compartments of 14 different tissues to study the degree of ubiquity present among tissues.

Methods: The Human MitoCarta Database was used to create our library of proteins, and their specific tissue localization. Only 14 tissue locations were considered in this database and they include: cerebrum, cerebellum, brainstem, kidney, liver, heart, skeletal muscle, adipose, stomach, small intestine, large intestine, testis and placenta. Next, we filtered out the proteins only found in the M and IMS compartments by determining their sub-cellular localization using the GeneCard Database.

Results: Data analysis showed that 75.44% M proteins are ubiquitously shared between all 14 tissues. There are only 3.51% of known soluble proteins found in only 8 or fewer tissues. In contrast, to the IMS proteins, only 16.67% of known soluble proteins of the IMS are found in all 14 tissues. We must acknowledge that 19.44% of soluble proteins are found in 13 of the 14 tissues. However, this still leaves 63.89% of IMS proteins which are unique to a varying number of tissues. 13.90% exist in 5 or fewer tissues, in contrast to 2.63% in the M, which contributes to the high variability of IMS proteins. Homologous protein analysis of IMS proteins showed that 14 tissue proteins shared the most proteomic

homology with small intestine (95.7%), testes (95.9%) and placenta (96.8%) tissues while the least homology was shared with skeletal muscle (61.8%) tissue.

Conclusion: Our data shows that there is a considerable proteome difference between tissues in the IMS compartment of the mitochondria, but this difference is significantly less in the M compartment. The proteins found in the M compartment seem to be more ubiquitous, implying the majority of these proteins are essential to the functioning of most tissues. This ubiquity in the M compartment further highlights the peculiarity of the high variability in the IMS proteomic milieu. More experimentation and analysis needs to be done to determine whether this compositional variability plays a role in the diversity of energetic capacity and demand seen among tissues.

P1352

Board Number: B696

FoxO1 switches energy metabolism and stimulates mitochondrial fission in myocardial insulin resistance.

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Cardiomyocytes are highly differentiated cells responsible of myocardial contraction. They have large requirements of energy making them highly sensitive to changes in intracellular energy metabolism. Mitochondria are crucial for the production of ATP, and their morphology and function are regulated by the dynamic processes of fusion and fission. We have recently shown that mitochondrial morphology and metabolism are regulated by insulin in cultured cardiomyocytes (Parra et al. Diabetes, 2014), suggesting that the balance between demand and energetic support regulates mitochondrial morphology. Despite this apparent relationship between a dysregulation of mitochondrial dynamics and myocardial energy deficiency, the precise mechanism of how this contributes to diabetic cardiomyopathy is still unclear. The transcription factor Forkhead box protein O1 (FoxO1) is negatively regulated by insulin and connects multiple pathways associated to energy homeostasis. The cardiomyocyte-specific deletion of FoxO1 prevents loss of cardiac function triggered by high fat diet, preserving cardiomyocyte insulin responsiveness (Batripolu et al. J Clin Invest, 2012). In the present work we evaluate the role of FoxO1 on cardiomyocyte mitochondrial morphology and metabolism in basal and under down regulation of insulin signaling conditions. To this end, a constitutively active FoxO1 (FoxO1CA-GFP) was overexpressed in primary cardiomyocytes or silenced with a specific siRNA. We evaluated the number and mean mitochondrial volume through 3D-reconstruction of the mitochondria network using ImageJ software. Our results showed that FoxO1CA induces mitochondrial fission and decreases mitochondrial metabolism without altering mitochondrial mass. In spite of a

reduction in expression of most of the mitochondrial dynamics related genes (Opa-1, Mfn-2, Drp-1 and Fis-1) by expression of FoxO1CA we not observed significant changes in its protein levels. Under this condition, FoxO1 was mainly present in the nuclei of cardiac cells transduced with FoxO1CA. In addition, FoxO1CA reduces the mitochondrial membrane potential, oxygen consumption and ^3H -2D-glucose uptake with a lightly increase in ATP levels. These metabolic effects could be related with a decreased expression in crucial metabolic gene expression (HK-II, mCTP-1, UCP-2 among others). Finally, chronic exposure of cardiomyocytes to insulin reduces the mitochondrial metabolism and increases the fission pattern of mitochondrial network. Collectively our results suggest that FoxO1 stimulates a metabolic switch and mitochondrial fission in cardiomyocytes and could mediate those metabolic changes observed in myocardial insulin resistance.

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P1353

Board Number: B697

Molecular regulation of receptor-mediated mitochondrial autophagy.

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Mitochondria control both cell's life and death. To ensure the well-being of the cell, mitochondrial quality must be tightly monitored through a mechanism of mitochondrial autophagy or mitophagy, which selectively removes damaged or unwanted mitochondria. We recently have identified that mitochondrial outer membrane protein FUNDC1 harbors LC3-interacting region (LIR) and interacts with LC3 to mediate mitophagy upon hypoxic treatment. Under normoxic condition, FUNDC1 is highly phosphorylated by Src kinase and CK2 at Try18 and Ser13, respectively. Mitochondrial stresses and hypoxic treatment lead to the dephosphorylation of FUNDC1 that has higher affinity with LC3. Importantly, we have identified that mitochondrially localized phosphatase PGAM5 is able to dephosphorylate FUNDC1 at Ser-13. The phosphatase activities of PGAM5 is inhibited through its interaction with Bcl-xL, and the degradation of Bcl-xL could lead to the activation of PGAM5 for mitophagy. Our results delineate the signaling pathway linking mitochondrial stresses and hypoxia towards the activation of mitophagy.

Keywords: Mitochondrial autophagy, mitochondrial stress, Hypoxia, FUNDC1, Bcl-xL

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Signaling Receptors (RTKs and GPCRs) 2

P1354

Board Number: B801

Regulation of Amyloid Precursor Protein Processing by Serotonin Signaling.

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Proteolytic processing of the amyloid precursor protein (APP) by the β - and γ -secretases releases the amyloid- β peptide (A β), which deposits in senile plaques and contributes to the etiology of Alzheimer's disease (AD). The α -secretase cleaves APP in the A β peptide sequence to generate soluble APP α (sAPP α). Upregulation of α -secretase activity through the 5-hydroxytryptamine 4 (5-HT4) receptor has been shown to reduce A β production, amyloid plaque load and to improve cognitive impairment in transgenic mouse models of AD. Consequently, activation of 5-HT4 receptors following agonist stimulation is considered to be a therapeutic strategy for AD treatment; however, the signaling cascade involved in 5-HT4 receptor-stimulated proteolysis of APP remains to be determined. Here we used chemical and siRNA inhibition to identify the proteins which mediate 5-HT4d receptor-stimulated α -secretase activity in the SH-SY5Y human neuronal cell line. We show that G protein and Src dependent activation of phospholipase C are required for α -secretase activity, while, unexpectedly, adenylyl cyclase and cAMP are not involved. Further elucidation of the signaling pathway indicates that inositol triphosphate phosphorylation and casein kinase 2 activation is also a prerequisite for α -secretase activity. Our findings provide a novel route to explore the treatment of AD through 5-HT4 receptor-induced α -secretase activation.

P1355

Board Number: B802

Differential Function of Type I Receptors in BMP Heterodimer-assembled Complexes.

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Bone morphogenetic proteins (BMPs) are important biopharmaceuticals in the treatment of skeletal conditions and in applications of tissue engineering. In these clinical applications and throughout vertebrate development, BMPs provide differentiation and growth cues in a time- and dose-dependent manner. The earliest function of BMPs is to pattern the embryonic dorsoventral axis. In the zebrafish gastrula, high BMP signaling levels arise ventrally whereas BMP antagonists, emanating from the dorsal organizer, attenuate signaling dorsally. Here BMP signaling requires two ligands, Bmp2b and Bmp7a, functioning exclusively as a heterodimer, and their corresponding type I receptors, Alk3/6 and Alk8. Why

BMP heterodimers function as the obligate ligand, while BMP homodimers, although present, fail to signal is fundamental to the BMP signaling mechanism and relevant to biopharmaceutical applications. Furthermore, evidence supports the role of BMP heterodimers in at least three other developmental pathways in zebrafish. Our goal is to elucidate the mechanism for the obligate function of BMP heterodimers in DV patterning. One model for the obligatory function of BMP heterodimers is that BMP antagonists preferentially block BMP homodimers. To test this model, we depleted BMP antagonists in embryos devoid of BMP heterodimers. If antagonists block BMP homodimers, we expect BMP homodimers to signal when BMP antagonists are absent. Intriguingly, signaling was not observed in embryos lacking BMP antagonists and BMP heterodimers indicating that BMP antagonists do not preferentially block BMP homodimers in vivo. When overexpressed, however, Bmp2b homodimers can signal, but unexpectedly, they require Alk8, the Bmp7a associated receptor. This suggests that BMP heterodimers prevail by virtue of assembling two different classes of type I receptor in a signaling complex. To examine the function of each class of type I receptor in the signaling complex, we generated a series of kinase defective receptor mutants. Interestingly, embryos were patterned by unactivatable Alk3a receptors. In contrast, Alk8 kinase activity was required for patterning. These results suggest an unequal contribution of from each receptor in the signaling complex. The BMP signaling pathway is heavily studied yet the careful consideration of heterodimer activity is understudied and represents a significant gap in our knowledge of the BMP signaling mechanism, which we are elucidating here.

P1356

Board Number: B803

Role of PARKIN/PARK2 in Bone resorptive capacity of osteoclasts.

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Dysfunction of PARKIN is associated with the progression of parkinsonism, which can contribute to a progressive systemic skeletal diseases characterized by low bone mineral density. However, the possible role of PARKIN in bone metabolism is not elucidated. In this study, we observed that PARKIN deficient mice exhibit a lower bone volume than wild-type (WT) mice from the micro-CT analysis. To determine whether the osteoporotic phenotype observed these mice is related by the activity of osteoclast (OC) or osteoblast (OB), we compared the mRNA expression level of PARKIN in bone-related cells. We found that PARKIN was markedly increased in mature OC and OB compared with its progenitor cells, respectively. Knock-down of PARKIN using siRNA significantly enhanced both OC differentiation and bone resorbing activity on dentin without any changes in OB differentiation. PARKIN deficient mice also showed higher rates of OC differentiation and subsequent bone resorbing activity than WT mice. Interestingly, we found that PARKIN co-localized with a microtubule and altered microtubule dynamics in mature OC, suggesting a negative regulatory role of PARKIN in osteoclastic bone resorption. Collectively, these results indicate that PARKIN can alter microtubule dynamics in mature OC and contribute to the maintenance of bone turnover balance by regulating OC functions.

P1357**Board Number: B804****Arhgap21 modulates CXCR7 in bone marrow of homozygous knockout mice.**R.C. Melo¹, C.L. Bigarella¹, K.V. Ferro¹, L. Ricon¹, J.M. Xavier², S.T. Saad²;¹Hematology and Hemotherapy Center, University of Campinas, Campinas, Brazil, ²Hematology and Hemotherapy Center, University of Campinas, UNICAMP, Campinas, Brazil

ARHGAP21 is a member of the RhoGAP family, which are negative regulators of RhoGTPases promoting the hydrolysis of GTP-bound Rho proteins to GDP. RhoGTPases are important regulators of cytoskeleton rearrangements that have been described in controlling migration, adhesion, cell cycle and survival. ARHGAP21 has been described to interact with PKC ζ and FAK which are downstream proteins of the CXCL12/CXCR4 pathway. CXCL12 is a chemokine produced by bone marrow stromal cells that binds to CXCR4. CXCR4 is a receptor expressed, among other cells, in hematopoietic progenitor cells. The activation of CXCR4 by CXCL12 can induce many processes such as migration, adhesion and homing of hematopoietic progenitor cells. Heterozygous knockout mice for ARHGAP21 (Arhgap21^{+/-} mice), produced by our group, exhibit reduced migration, adhesion and homing of progenitor cells suggesting that ARHGAP21 might be a strong candidate to regulate the CXCL12/CXCR4 signaling. Recently, CXCR7 was identified as another CXCL12-binding receptor, but its contribution to CXCL12-mediated effects in hematopoietic cells is still poorly explored, even though the CXCR7 relationship with tumor progression in non-hematopoietic malignancies is well established. Given that migration, adhesion and homing are altered in Arhgap21^{+/-} mice and that there is little information regarding CXCR7 in hematopoiesis, we investigated its expression in bone marrow cells of Arhgap21^{+/-} mice. In addition, we also investigated the expression of CXCR4 in these cells and CXCL12 levels in the supernatant cell. Bone Marrow was isolated from mice femurs, tibias and humerus by crushing the bones. Cells were passed through a 0.40 μ M cell strainer and red blood cells were lysed with lysis buffer solution. Afterwards, CXCR7 and CXCR4 mRNA expression was analyzed by Real-time PCR (normalized by HPRT) in bone marrow samples of 20 Arhgap21^{+/-} and 17 wild-type mice. The CXCL12 dosage was analyzed by ELISA. CXCR7 was significantly lower expressed in samples from heterozygous compared to wild-type mice (1.09 [0.24-2.49] versus (vs.) 0.60 [0.00-1.78]; $p=0.0161$, *Mann-Whitney* test). No difference in CXCR4 expression neither in the levels of CXCL12 was observed. These results suggest that, in bone marrow, CXCR7 is modulated by ARHGAP21 and could play a role in the impaired homing observed in Arhgap21^{+/-} mice.

P1358**Board Number: B805****The role of Thrombin in proliferation of human Müller Glial Cells.**I. Lee Rivera¹, M.G. Carranza-Pérez¹, E. López-Hernández¹, A. López-Colomé¹;¹Instituto De Fisiologia Celular UNAM, Mexico DF, Mexico

The breakdown of the blood–retina barrier (BRB) by trauma, ischemia, anoxia or metabolic imbalance has been shown to be a key factor for the progress of ocular proliferative diseases such as Proliferative Vitroretinopathy (PVR), retinal neovascularization and Diabetic retinopathy. Müller glial cells (MCs) play a central role in BRB maintenance: in pathological conditions, they also act as modulators of inflammatory responses. Depending on the severity of the challenge, MCs activate a proliferation program that, in mammalian retina, generates fibrotic scars that contribute to the formation of contractile epi-retinal membranes, which detach the retina, with the consequent loss of vision. Therefore, it is clinically relevant to understand the mechanisms involved in MCs proliferation. The pro-inflammatory Serine-protease Thrombin has been shown to participate in a myriad of cellular processes including proliferation and migration. Thrombin actions are mediated by the activation of G-protein coupled Protease Activated Receptors (PARs 1-4) through the cleavage of the N-terminal domain. The purpose of this study was to investigate the effect of thrombin on the proliferation of human MCs using the MIO-M1 cell line. We first demonstrated that human MCs express all four classes of PARs, both at the mRNA and protein level. Functionally, our results show that Thrombin dose-dependently stimulates MC proliferation by 44%, with a calculated EC_{50} of 0.86 nM. In contrast to our previous findings showing maximal Thrombin-induced RPE cell proliferation at 24 hours post-stimulation, MCs peak proliferation required sustained Thrombin treatment for 72 hours. These results suggest that duration of the stimulus influences the composition and possibly, contractility of epi-retinal membranes. The specificity of Thrombin action was demonstrated by the prevention of proliferation using the thrombin chelator Hirudin and its catalytic inhibitor PPACK in MTS reduction and wound healing assays. MAPKs have been shown to mediate c-fos expression, Cyclin D1 accumulation and RPE cell proliferation. To investigate the possible signaling mechanisms involved in thrombin-induced human MC proliferation, we analyzed Erk1/2 phosphorylation/activation. Results demonstrate that, as previously shown for RPE cells, MEK/Erk signaling pathway is activated by thrombin in human MCs. Since thrombin induces the proliferation of RPE and MCs, the main cellular components of epi-retinal membranes, the present results suggest that this protease may play an important role in proliferative blinding diseases such as PVR and gliosis. This study was partially supported by grants from CONACyT (176347) and PAPIIT-UNAM. We acknowledge the donation of MIO-M1 cells by Dr. GA Limb (University College London, UK).

P1359**Board Number: B806****A pivotal role for non-muscle myosin II in the negative regulation of signal transduction mediated by the B-cell antigen receptor.**W. Song¹, C. Liu², M.I. Lazzaro³;¹Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD, ²Cell Biology and Molecular Genetics, University of Maryland, College Park, MD, ³Univ Maryland, College Park, MD

B-lymphocytes rely on B-cell antigen receptor (BCR) to sense pathogenic substances and initiate B-cell-mediated antibody responses. Upon binding to antigen, the BCR initiates signaling cascades, which can be up and down regulated based on the nature of antigen and the levels of antibody-antigen immune complexes. BCR activation requires actin-dependent receptor oligomerization into microclusters. We have recently shown that besides signaling initiation and amplification, actin remodeling is critical in the attenuation of BCR signaling, by driving B-cell contraction on the antigen-presenting surface and the coalescence of BCR microclusters into a central cluster. However, the cellular mechanism underlying the BCR-actin interplay during signal attenuation is unknown. In this study, we demonstrate that BCR activation induces the activation and recruitment of non-muscle myosin II (NMII) to the B-cell surface that interacts with antigen-presenting surface, first to BCR microclusters and later to the outer rim of the BCR central cluster. The NMII recruitment is dependent on SH2 domain-containing inositol 5-phosphatase (SHIP-1), as BCR-induced recruitment is blocked in SHIP-1^{-/-} B-cells and enhanced when the kinetics and magnitude of SHIP activation are increased by the colligation of the BCR with the inhibitory co-receptor Fc gamma receptor IIB (FcgammaRIIB) by immune complexes. Inhibition of NMII activation or motor activity by the Rho-associated protein kinase inhibitor or blebbistatin causes an increase in B-cell spreading on the antigen-presenting surface, a delay in B-cell contraction, and inhibition of BCR central cluster formation, consequently leading to increased levels of tyrosine phosphorylation in response to membrane-associated antigen. Furthermore, the inhibition of NMII releases B cells from the suppression of FcgammaRIIB on B-cell spreading, BCR clustering and tyrosine phosphorylation. These results suggest that NMII is a critical component of the negative regulation machinery in B cells. NMII functions downstream of SHIP and mediates the transition from actin-driven B-cell spreading to contraction, consequently controlling BCR clustering and signal attenuation.

This work has been supported by Maryland Stem Cell Research Fund to WS.

P1360**Board Number: B807****Evidence that TMEM184A functions as a receptor for heparin.**Y. Li¹, J.B. Slee^{2,3}, L.J. Lowe-Krentz¹;¹Biological Sciences, Lehigh University, Bethlehem, PA, ²Lehigh University, Bethlehem, PA, ³Pediatrics, Children's Hospital of Philadelphia, Philadelphia, PA

Besides its well-known role as an anticoagulant, heparin has also been revealed to have anti-proliferative effects on vascular smooth muscle cells (VSMCs). Heparin treatment of VSMCs has been shown to decrease ERK activity and the activation of down-stream transcription factors in addition to decreasing cell proliferation. In addition, VSMCs and endothelial cells have long been known to bind and take up heparin. Despite this evidence, the underlying mechanism(s) resulting in heparin effects on VSMC proliferation have remained unclear. Evidence supporting a cell surface receptor for heparin includes monoclonal antibodies that block heparin binding and mimic heparin effects in inducing PKG-dependent responses in VSMCs. Studies in our laboratory indicate that the protein product of TMEM184A is precipitated with these antibodies. Immunoprecipitation using our monoclonal antibodies that mimic heparin and commercial TMEM184A antibodies reveals apparently identical protein. Western blots of the immunoprecipitates stained by a second commercial TMEM184A antibody raised against an alternate epitope support the identification. Similar immunoprecipitation results are obtained with VSMCs and bovine aortic endothelial cells. Treatment of GFP-TMEM184A transfected VSMCs with rhodamine-heparin allows evaluation of heparin uptake. Confocal microscopy was used to identify early co-localization of the fluorescent labels and rhodamine-heparin uptake into vesicles coated with the GFP construct. These results suggest association between TMEM184A and its involvement in heparin internalization. Together these data provide evidence supporting the identification of TMEM184A as a heparin receptor on vascular cell membranes.

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P1361**Board Number: B808****Mechanistic Study of the TRPM2-Ca²⁺ Signaling in ROS induced Switch between Apoptosis and Autophagy.**J. Yue¹;¹Biomedical Sciences, City University of Hong Kong, Hong Kong, Hong Kong

It is well known that reactive oxygen species (ROS) regulates autophagy, and vice versa. Yet, the molecular entities underlying the interplay between ROS and autophagy remain elusive. Transient potential receptor melastatin-2 (TRPM2) is a Ca²⁺-permeable cation channel, and is mainly activated by intracellular adenosine diphosphate ribose (ADPR) in response to oxidative stress. Here we found that ROS activated TRPM2 for Ca²⁺ influx via ADPR to inhibit early autophagy induction, which ultimately led

to apoptosis in TRPM2 expressing cancer cell lines. On the other hand, ROS induced autophagy, not apoptosis, for cell survival in cancer cell lines which do not express TRPM2, and autophagy inhibition converted cells to apoptosis upon ROS treatment. Likewise, TRPM2 knockdown or inhibition rewired cells from apoptosis to autophagy for survival in response to ROS in TRPM2 expressing cells. ROS-induced Ca^{2+} influx via TRPM2 actually activated calmodulin-dependent protein kinase II (CaMKII) to phosphorylate Ser295 on Beclin1. Phosphorylated BECLIN1, in turn, decreased the association between BECLIN1 and VPS34, but induced the binding between Beclin1 and Bcl2. Moreover, ROS induced Ca^{2+} influx via TRPM2 markedly augmented intracellular ROS levels in a positive feedback loop, which subsequently oxidized CaMKII to enable CaMKII binding with BECLIN1. In summary, our data demonstrated that the TRPM2/ Ca^{2+} /CaMKII/Beclin1 cascade is the molecular switch between autophagy and apoptosis in response to ROS.

P1362

Board Number: B809

Basigin regulates spermatocyte apoptosis through extrinsic apoptotic pathway.

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Basigin (BSG), also known as cluster of differentiation 147 (CD147) or extracellular matrix metalloproteinase inducer (EMMPRIN), is a transmembrane glycoprotein that plays an important role in spermatogenesis. Spermatogenesis is a physiological process tightly regulated by germ cell proliferation and apoptosis. Previous study has shown that BSG null mutant male mice are infertile. The percentage of apoptotic germ cell increases, and spermatogenesis is arrested in BSG knockout mice. Functional Blocking BSG with anti-BSG induces apoptosis in spermatocytes. However, it is still elusive that how BSG is involved in germ cell fate regulatory network. In this study, we aim to clarify the molecular mechanism underlying BSG-mediated germ cell survival/apoptosis with the mouse spermatocyte cell line GC-2 in vitro and mouse testicular germ cells in vivo. We found that the cleaved caspase 8 was dramatically increased in anti-BSG treated GC-2 cells and BSG-immunodepleted mice testis, indicating that anti-BSG induced extrinsic apoptosis in germ cells. Indeed, the expression of TNF- α and Fas, the stimulators of extrinsic apoptosis, were significantly increased in BSG-immunodepleted testis. Meanwhile, NF κ B signaling was down-regulated in both anti-BSG treated GC-2 cells and BSG-immunodepleted mice testis. These findings support an important role of BSG in regulating germ cell apoptosis.

P1363**Board Number: B810****Trehalose promotes selective secretion of Insulin-like peptide 3 through an adipokinetic hormone relay in *Drosophila*.**J. KIM¹, T. Neufeld^{1,2};¹Genetics Cell Biology and Development, University of Minnesota, Minneapolis, MN, ²Genetics, Cell and Developmental Biology, University of Minnesota, Minneapolis, MN

Secreted ligands of the insulin family play dual roles in promoting cell growth and maintaining organismal sugar homeostasis. Release of insulin is tightly regulated in response to levels of circulating nutrients, but the mechanisms by which insulin producing cells (IPCs) coordinate their response to distinct nutrient signals and other hormonal pathways during development are unclear. Here we show that regulation of insulin secretion in *Drosophila* larvae has been segregated into distinct branches: circulating sugars selectively promote the release of *Drosophila* insulin-like peptide 3 (Dilp3), whereas amino acids selectively promote secretion of Dilp2. Dilp3 is uniquely required for sugar-mediated activation of insulin signaling and suppression of autophagy in the larval fat body, a central regulator of Dilp secretion. We demonstrate that sugar levels are not sensed directly by the IPCs, but rather by the adipokinetic hormone (AKH)-producing cells of the corpora cardiaca, and that AKH signaling is required in the IPCs for sugar-dependent Dilp3 release. Thus, the IPCs integrate multiple cues to regulate selective secretion of distinct insulin subtypes, thereby maintaining homeostasis under varied nutrient conditions.

P1364**Board Number: B811****Surface Adhesion and Proliferation of 3T3-L1 Pre-Adipocytes Exhibit Sensitivity To Melanin-Concentrating Hormone: High Dose Effects Do Not Predict Low Dose Effects.**M. Cerasaro¹, C. King¹, L.B. Cook¹;¹Biology, SUNY Coll-Brockport, Brockport, NY

Melanin-concentrating hormone (MCH) participates in a variety of physiological processes, including regulation of sleep-wake cycles, appetite and energy expenditure. Although a great deal of research is focused on MCH signaling in brain, we can't rule out the potential significance of MCH receptor targets in tissues throughout the body including the gut, pancreas and adipose stores. We recently reported that MCH facilitates pre-adipocyte migration in scratch wound assays, providing evidence that MCH signaling may actually participate in the development of adipose tissue. Interestingly, in addition to cell migration, mitotic expansion of cells is also expected during the differentiation process. The purpose of this continuing study was to test the hypothesis that MCH influences the mitotic expansion of murine 3T3-L1 pre-adipocytes in culture. A crystal violet assay is simple and useful for obtaining quantitative

information about the relative number of cells adhering to multi-well plates. Crystal violet stains DNA and, upon cell lysis, the dye taken up by the monolayer can be quantitated in a spectrophotometer. We cultured 3T3-L1 pre-adipocytes in the presence of MCH for 6 days and discovered that incubation with just 1 nM MCH resulted in significantly more cells than control and neither 10 nM nor 100 nM had any measurable effect. To determine if this increase in cell number was influenced by changes in cell adherence to the culture dish, we performed a time-course growth assay where cells were incubated in 1 nM MCH for up to 72 hours. Using a hemacytometer to count cells in the media as well as adhering to the plate, we determined that low dose MCH results in less cells suspended in the media and more cells adhering to the plate, but that compared to control, the overall number of cells in MCH-treated wells significantly outnumbered those untreated. In conclusion, we have uncovered a previously unknown level of sensitivity in MCH receptor signaling, with 1 nM MCH facilitating adherence and mitotic expansion of 3T3-L1 preadipocytes that is undetectable with 100 nM MCH. Future experiments are aimed at determining whether differentiated 3T3-L1 adipocytes exhibit similar sensitivity to MCH and whether the observed effects translate to human pre-adipose and adipose cells as well.

P1365

Board Number: B812

The Contribution of Melanoregulin to Microtubule-Associated Protein 1 Light Chain 3 (LC3) Associated Phagocytosis.

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The degradation of shed photoreceptor outer segments by the retinal pigment epithelium (RPE) is necessary for photoreceptor renewal, function and survival. Phagocytic uptake, phagosome maturation and subsequent degradation of photoreceptor outer segment (OS) content require a coordinated series of processes regulated by several intracellular proteins. One such protein melanoregulin (MREG) is necessary for the complete digestion of OS; loss of MREG results in incomplete degradation of opsin positive phagosomes as well as lipofuscin accumulation over time. Here we explore the hypothesis that MREG-dependent processing of POS phagosomes links aspects of both autophagic and phagocytic processes in a pathway known as LC3-dependent phagocytosis (LAP). Opsin positive phagosomes were associated with LC3 as well as MREG both *in vivo* (by immuno-EM) and *in vitro* (by confocal imaging). The association of LC3 with OS was independent of nutrient deprivation mediated by rapamycin and dependent on the presence of Atg5. Phagosome maturation studies suggest that MREG is required for LC3 association with POS. Furthermore, the mechanism of MREG action is likely through an interaction with LC3, as determined by co-localization and immuno-precipitation studies. Lastly, the consequence of defective MREG-LC3 association results in the accumulation of cholesterol and 7-ketocholesterol. We posit that POS degradation is a complex process likely requiring convergence of the phagocytic and autophagic pathways in a process that involves LC3 and MREG.

P1366**Board Number: B813****Exploration of a novel signaling pathway implicated in the regulation of insulin secretion from β -cells.**G. Li¹, S. Shao¹, H. Xu¹, Y. Bee²;¹Dept of Clinical Research, Singapore General Hospital, Singapore, Singapore, ²Dept of Endocrinology, Singapore General Hospital, Singapore, Singapore

Insufficient insulin release and/or defective insulin action lead to the development of diabetes. Insulin secretion from islet β -cells is controlled by a complex signaling network. Lysophospholipids constitute a group of important lipid mediators, among which lysophosphatidic acid (LPA) exhibits multiple biological functions e.g. platelet aggregation, smooth muscle contraction and cell proliferation. LPA may evoke its effects through G-protein-coupled receptors followed by activation of various signaling pathways such as phospholipases C and D, and mitogen-activated protein kinase. LPA can be generated via hydrolysis of phosphatidic acid (PA) by phospholipase A1 (PLA1) and A2. This study investigated the possible role of a cytosolic PLA1 with a strong preference for PA (PA-PLA1) in insulin-secreting cells. The activity of this PA-PLA1 was originally identified in the supernatant fraction of bovine testis and brain. We observed high expression of PA-PLA1 in isolated rat islets and also in insulin-secreting INS-1 β -cells as determined by real-time RT-PCR and immuno-blotting. By contrast, PA-PLA1 expression was undetectable in isolated pancreatic acinar cells. The function of PA-PLA1 in β -cells was assessed after its knockdown by small RNA interference. Transfection of INS-1 cells with a mixed pool of 3 siRNA oligos targeting PA-PLA1 mRNA for 3 days reduced its expression by 80%. PA-PLA1 knockdown caused a decrease of LPA levels in INS-1 cells as determined by thin-layer chromatography, but had no effect on the cell proliferation as assessed by flow cytometry. Glucose metabolism was lowered after PA-PLA1 knockdown as measured by a tetrazolium-based MTS assay. Importantly, high glucose (11.1-22.2 mM)-stimulated insulin secretion was significantly reduced by >75%, with a severer inhibition on the late phase secretion. Moreover, insulin secretion induced by the sulfonylurea glibenclamide and by membrane potential depolarization with high K⁺ was markedly diminished. However, PA-PLA1 knockdown did not alter glucose-stimulated insulin release in the presence of forskolin and GLP-1 which can increase cAMP levels. In parallel, PA-PLA1 knockdown inhibited glucose-induced increase of cAMP levels but an addition of forskolin or GLP-1 could rescue such inhibition. Our data suggest that PA-PLA1 might be a novel lipase which play an important role in the regulation of insulin secretion in β -cells via cAMP-implicated signaling pathway.

P1367**Board Number: B814****Endocytosis and Vacuolar Degradation of the Yeast Cell Surface Glucose Sensors Rgt2 and Snf3.**A. Roy¹, J. Kim²;¹Biochemistry and Molecular Medicine, George Washington University, Washington, DC, ²George Washington University, Washington, DC

Sensing and signaling the presence of extracellular glucose is crucial for the yeast *Saccharomyces cerevisiae* because of its fermentative metabolism, characterized by high glucose flux through glycolysis. The yeast senses glucose through the cell surface glucose sensors Rgt2 and Snf3, which serve as glucose receptors that generate the signal for induction of genes involved in glucose uptake and metabolism. Rgt2 and Snf3 detect high and low glucose concentrations, respectively, perhaps because of their different affinities for glucose. Here, we provide evidence that the glucose sensors are removed from the plasma membrane through endocytosis and targeted to the vacuole for degradation upon glucose depletion. The turnover of the glucose sensors is inhibited in endocytosis defective mutants, and the sensor proteins with a mutation at their putative ubiquitin-acceptor lysine residues are resistant to degradation. Of note, the low affinity glucose sensor Rgt2 remains stable only in high glucose grown cells, and the high affinity glucose sensor Snf3 is stable only in cells grown in low glucose. In addition, constitutively active glucose sensors do not undergo endocytosis, whereas signaling defective sensors are constitutively targeted for degradation, suggesting that the actively signaling state of the glucose sensors is protected from endocytosis. Taken together, our findings demonstrate that the amount of glucose available dictates the stability of the two glucose sensors with different affinities for glucose, enabling yeast cells to maintain glucose sensing activity over a wide range of glucose concentrations.

P1368**Board Number: B815****A Novel Transfluor BacMam Reagent for Monitoring GPCR Activation.**A. Forsyth¹, P. Gedraitis¹, J.A. Hesley¹, E.F. Cromwell¹;¹Molecular Devices, Sunnyvale, CA

G-protein-coupled receptors (GPCR) participate in many disease pathways and represent the largest family of therapeutic targets. Thus, great investments are made to discover drugs modulating GPCR-mediated events. Transfluor is a functional high-content imaging based GPCR assay that measures redistribution of cytosolic beta-arrestin following binding to an activated GPCR and can be used for drug screening. By attaching a fluorescent label to beta-arrestin, the location of the receptor-arrestin complex can be monitored. Since desensitization only occurs with an activated receptor, monitoring beta-arrestin translocation and subsequent receptor recycling provides a method to detect the activation of any GPCR. BacMam viruses are modified baculoviruses that contain mammalian expression cassettes for viral gene delivery and transient expression in mammalian cells. Here we show the

applicability of a novel BacMam-based reagent for performing Transfluor assay. A virus engineered to express the labeled beta-arrestin protein can be used to transduce cell lines expressing the GPCR of interest. Here we show data generated, using the ImageXpress Micro XLS high-content imaging system, from a CHOK1 cell line stably expressing the Angiotensin II receptor type 1 (AT1). We observed a diffuse cytoplasmic GFP fluorescence signal in unstimulated cells, that became punctate upon stimulation with receptor-specific agonists, indicative of the relocation of the receptor-arrestin complex to clathrin-coated pits on the cell membrane and then to endocytic vesicles. This response was quantified by the MetaXpress Imaging Software's Transfluor application module and allowed the generation of dose-response curves and EC₅₀ values. In addition, inhibition of receptor trafficking by antagonists was also demonstrated. This combination of BacMam and Transfluor technology allows a flexible, versatile and universal GPCR cell-based assay able to screen for GPCR ligands regardless of the interacting G-Protein or signaling pathway utilized.

P1369

Board Number: B816

G-protein beta-gamma regulation of Golgi to cell surface transport.

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Non-canonical G-protein signaling has been shown to regulate intracellular transport and secretory pathways at the Golgi. G-protein beta-gamma localizes to the trans-Golgi network, activating effector phospholipase C to increase local diacylglycerol and recruit members of a kinase signaling cascade. Subsequent activation of protein kinase C results in phosphorylation and activation of protein kinase D (PKD). Numerous PKD substrates are phosphorylated at the TGN, resulting in recruitment of vesicle fission machinery and consequent fission of vesicles destined for the plasma membrane. While basolaterally-targeted (BL) or secretory vesicle release is known to be downstream of PKD activation, apically-targeted (AP) vesicle release from the Golgi occurs via a PKD-independent pathway. A similar cargo selectivity for beta-gamma has not been tested. Here, we generate GRK2ct variants that inhibit beta-gamma in a spatial or temporal manner to test regulation of AP and BL cargo trafficking. We find that a lipid-association mutant of GRK2ct fails to inhibit beta-gamma function at the Golgi, but forced targeting to the Golgi recovers inhibition of beta-gamma. We use this lipid-association mutant of GRK2ct to generate an inducible system of recruitment of GRK2ct variants to distinct subcellular locations, towards the goal of inhibiting solely Golgi-localized beta-gamma signaling. We aim to use these tools to separate different signaling pools of beta-gamma, depending on sub-cellular localization. We follow model cargo trafficking via transport of temperature-sensitive cargoes to the plasma membrane, as well as secretory cargo delivery to the media. We find that beta-gamma inhibits only BL cargo delivery, in both PM-destined and secretory cargo. The results indicate that beta-gamma functions upstream of PKD as a regulator of BL (but not AP) vesicle transport from the Golgi to the cell surface.

P1370**Board Number: B817****Up-regulation of N-cadherin by collagen I-activated DDR1 requires Shc1.**H. Huang¹, R.A. Svoboda², J. Saowapa², K.R. Johnson^{2,3};¹Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE, ²Oral Biology, University of Nebraska Medical Center, Omaha, NE, ³Fred Pamela Buffett Cancer Center, University of Nebraska Medical Center, Omaha, NE

Pancreatic ductal adenocarcinomas are highly malignant cancers, characterized by extensive invasion into surrounding tissues, metastasis to distant organs at a very early stage, and a limited response to therapy. One of the main features of pancreatic ductal adenocarcinomas is desmoplasia, which leads to extensive deposition of collagen I. Our lab has demonstrated that collagen I can induce epithelial-mesenchymal transition (EMT) in pancreatic cancer cells. A hallmark of EMT is an increase in the expression of a mesenchymal cadherin, N-cadherin. Our previous studies have shown that up-regulation of N-cadherin can promote tumor cell invasion, and collagen I-induced EMT is through two collagen receptors $\alpha 2\beta 1$ integrin and discoidin domain receptor 1 (DDR1). DDR1 is a receptor tyrosine kinase widely expressed during embryonic development and in many adult tissues. It is also highly expressed in many different cancers. However, the role of DDR1 in pancreatic cancer is largely unknown. In the collagen I-induced up-regulation of N-cadherin signaling pathway, we have shown that focal adhesion kinase (FAK)-related protein tyrosine kinase (Pyk2) is downstream of DDR1. In this study, we found that isoform b of DDR1 is responsible for collagen I-induced up-regulation of N-cadherin and that it is Tyrosine513 of DDR1b that is involved. Knocking down Shc1, which binds to Tyrosine513 on DDR1b, eliminates N-cadherin up-regulation. Interestingly, we also found Shc1 directly binds to Pyk2 and that collagen I-DDR1b signaling induces the phosphorylation of Tyrosine402 on the Pyk2 that is associated with Shc1.

P1371**Board Number: B818****Micro-patterned surfaces as a superior tool for the quantitative analysis of protein-protein interactions in living cells.**P. Lanzerstorfer¹, Y. Yoneyama², U. Mueller¹, G. Kreindl³, S. Takahashi², O. Hoeglinger¹, M. Buenemann⁴, G. Schuetz⁵, J. Weghuber¹;¹School of Engineering and Environmental Sciences, University of Applied Sciences Upper Austria, Wels, Austria, ²Department of Animal Sciences, The University of Tokyo, Tokyo, Japan, ³EV Group GmbH, St. Florian/Inn, Austria, ⁴Institut für Pharmakologie und Klinische Pharmazie, Philipps-Universität Marburg, Marburg, Germany, ⁵Vienna University of Technology, Institute of Applied Physics, Vienna, Austria

The plasma-membrane of living cells is a major organelle for cellular signaling cascades. To warrant the diverse functions of a cell, communication between the cytoplasm and the extracellular space is crucial.

Thus, membrane-localized proteins activated by various messenger molecules are essential to transmit signals into the cell. Defective regulation of these signaling cascades may lead to cell death or uncontrolled proliferation. An important issue is the interaction of these membrane proteins with cytosolic proteins. To analyze such interactions we have recently introduced a method based on the combination of micro-structured surfaces and TIRF microscopy. This technique was developed to detect and quantify also weak or short-lived protein-protein interactions in live cells. In a first attempt we used our assay to validate the efficacy of medically relevant receptor tyrosine kinase (RTK) modulators. Bait-EGF/Insulin/IGF1 receptor molecules were forced into microscopic domains on the surface of living cells, while monitoring co-recruitment of fluorescent intracellular prey molecule such as Grb2/IRS1-3, respectively. HeLa cells expressing the prey Grb2 enabled us to quantify the EGFR-Grb2 interaction. Pretreatment with pharmacologically active ingredients used for the treatment of human cancers significantly reduced the inducibility of the signaling system. In addition, we performed fluorescence recovery after photobleaching (FRAP) experiments with RKT class II receptors (IR and IGFR) and different cytosolic insulin receptor substrate (IRS) proteins. Our results indicate prominent differences in the interaction strength of IRS1 and IRS2 to the IR/IGF1-R, compared to the one of IRS3. In a second attempt we studied the interaction of the G-protein coupled receptor β 2-adrenoceptor (β 2AR) with arrestin-3. By measuring arrestin-3 recruitment and the stability of arrestin-3-receptor complexes in real time using FRAP analysis on micro-patterned surfaces, we could demonstrate that arrestin-3 dissociates quickly, and almost completely from the β 2AR, whereas the interaction with a hyper-phosphorylated β 2AR was significantly prolonged. Finally, we investigated the activation of the EGFR by transactivation via β 2AR. This process of transactivation is highly complex and appears to depend on a number of factors including the cell line, the type and class of the GPCR and the cellular environment. Using the micro-patterning approach we studied the localization and dynamics of key molecules involved in β 2AR-EGFR transactivation. An important issue which we are currently addressing is the production of a micro-structured and functionalized multiwell plate. This development step will set a milestone in terms of throughput rates and increase the number of potential users interested in our methodology.

1. Lanzerstorfer, P. et al., PLOS ONE. 2014.
2. Lanzerstorfer, P. et al., Basic Methods in Protein Purification and Analysis. 2013.
3. Weghuber, J. et al., Analytical and Bioanalytical Chemistry. 2010.
4. Weghuber, J.; et al., Methods Enzymol. 2010.
5. Schwarzenbacher, M. et al., Nature Methods. 2008.

P1372**Board Number: B819****Reactome Knowledgebase: Exploring biological pathways and networks.**R.A. Haw¹, R. Team¹, H. Hermjakob², P. D'Eustachio³, L. Stein¹;¹Informatics and Bio-computing, OICR, Toronto, ON, ²Proteomics Services, EBI, Hinxton, United Kingdom,³Departments of Biochemistry and Molecular Pharmacology , NYUMC, New York, NY

Modern health initiatives and drug discovery are focused increasingly on targeting diseases that arise from perturbations in complex cellular events. Consequently, there has been a tremendous effort in biological research to elucidate the molecular mechanisms that underpin normal cellular processes. A reaction-network pathway knowledgebase is the tool of choice for assembling and visualizing the “parts list” of proteins and functional RNAs, as a foundation for understanding cellular processes, function and disease. The Reactome Knowledgebase (www.reactome.org) is a publicly accessible, open access bioinformatics resource that stores full descriptions of human biological reactions, pathways and processes. Curated pathway knowledgebases, like Reactome, are uniquely powerful and flexible tools for extracting biologically and clinically useful information from the flood of genomic data. Specific features of Reactome support the visualization of interactions of many gene products in a complex biological process, and the application of bioinformatics tools to find causal patterns in expression data sets. To maximize Reactome’s coverage of the genome, we have supplemented curated data with a conservative set of predicted functional interactions (FI), roughly doubling our coverage of the translated genome. We have developed a Cytoscape app called “ReactomeFIViz”, which utilizes this FI network to assist biologists to perform pathway and network analysis to search for gene signatures from within gene expression data sets or identify significant genes within a list. Pathway and network-based tools for building and validating interaction networks derived from multiple data sets will give researchers substantial power to screen intrinsically noisy experimental data in order to uncover biologically relevant information.

Signaling from the PM/Cytoplasm to the Nucleus**P1373****Board Number: B820****SH2B1 β Interacts with STAT3 and Enhances Fibroblast Growth Factor 1-Induced Gene Expression during Neuronal Differentiation.**K. Chen¹, L. Chen¹;¹Institute of Molecular Medicine, National Tsing Hua University, Hsinchu, Taiwan

Neurite outgrowth is an essential process during neuronal differentiation as well as neuroregeneration. Thus, understanding the molecular and cellular control of neurite outgrowth will benefit patients with neurological diseases. We have previously shown that overexpression of the signaling adaptor protein

SH2B1 β promotes fibroblast growth factor 1 (FGF1)-induced neurite outgrowth. SH2B1 β also undergoes nucleocytoplasmic shuttling and regulates a subset of neurotrophin-induced genes. Although these findings suggest that SH2B1 β regulates gene expression, the nuclear role of SH2B1 β was not known. In this study, we show that SH2B1 β interacts with the transcription factor, signal transducer, and activator of transcription 3 (STAT3) in neuronal PC12 cells, cortical neurons, and COS7 fibroblasts. By affecting the subcellular distribution of STAT3, SH2B1 β increased serine phosphorylation and the concomitant transcriptional activity of STAT3. As a result, overexpressing SH2B1 β enhanced FGF1-induced expression of STAT3 target genes *Egr1* and *Cdh2*. Chromatin immunoprecipitation assays further reveal that, in response to FGF1, overexpression of SH2B1 β promotes the in vivo occupancy of STAT3-Sp1 heterodimers at the promoter of *Egr1* and *Cdh2*. These findings establish a central role of SH2B1 β in orchestrating signaling events to transcriptional activation through interacting and regulating STAT3-containing complexes during neuronal differentiation.

P1374

Board Number: B821

Decision-making in *C. elegans* chemotaxis to alkaline pH: Competition between two sensory neurons, ASEL and ASH.

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The monitoring of environmental and tissue pH is crucial for the survival of animals. The nematode *C. elegans* is a model organism suitable for the analysis of neural circuits that regulate animal behaviors. The animal is attracted to mildly alkaline pH, and avoids strongly alkaline pH. Our genetic dissection and Ca²⁺ imaging demonstrate that ASEL and ASH are the major sensory neurons responsible for the attraction to mildly alkaline pH and repulsion from strongly alkaline pH, respectively. In ASEL, a transmembrane guanylyl cyclase, GCY-14, is activated by environmental alkalization, and in turn, a cGMP-gated channel serves for Ca²⁺ influx into the sensory neuron (Ref. 1). In ASH, TRPV channels are found to be required for the neural activation upon stimulation with strongly alkaline pH (Ref. 2). To understand the animal's behavioral switch at molecular and cellular levels, we have also analyzed behaviors of mutants defective in ASEL and/or ASH under various alkaline pH, and have found that activities of ASEL and ASH compete each other for the behavioral switch. While mildly alkaline pH preferentially activates ASEL, strongly alkaline pH activates both ASEL and ASH, and ASH activity overrides the activity of ASEL (Ref. 3). Neural circuits responsible for this decision-making will be discussed. References: (1) Murayama et al., *Curr. Biol.* 23, 1007 (2013). (2) Sassa et al., *Neurosci. Lett.* 555, 248 (2013). (3) Murayama et al., *Commun. Integr. Biol.* 6, e26633 (2013).

P1375**Board Number: B822****Interleukin-22 enhances osteoclastogenesis by inducing the production of RANKL in osteoblast.**E. LEE¹, S. KIM², E. KIM², E. CHANG¹;¹Department of Biomedical Sciences, University of Ulsan College of Medicine, Seoul, Korea, ²Department of Biomedical sciences, University of Ulsan College of Medicine, Seoul, Korea

Interleukin-22 (IL-22) is a cytokine that mediates cellular inflammatory response. Although IL-22 was found to be increased in the patients with Rheumatoid arthritis (RA), the role of IL-22 in inflammatory bone disease has yet to be defined. We found that the level of IL-22 was elevated in inflammatory osteolytic condition characterized by trabecular bone destruction and high osteoclastogenesis. TNF α treatment elevated the IL-22 expression of osteoblasts. Exogenous IL-22 did not directly affect differentiation of osteoblast and osteoclast. However, when pOBs and precursor OCs were co-cultured, exogenous IL-22 significantly increased the number of tartrate-resistant acid phosphatase-positive multinucleated cells by increasing mRNA expression and protein secretion of RANK ligand (RANKL) in OBs. We also show that IL-22 significantly induces activation of ERK but not JNK in pOBs. IL-22 slightly induced phosphorylation of AKT on Thr308 as well as phosphorylation of STAT5 on tyrosine residues. Furthermore, IL-22 knockout mice were less susceptible to LPS-induced inflammation than were wild-type mice, as evidenced by their increased bone volume and increased trabecular thickness. The less severe form of bone loss in IL-22 $-/-$ mice might associate with production of RANKL in bone. These findings suggest that IL-22 is one of the pro-inflammatory mediators that contribute to the deteriorating osteolytic condition of inflamed bone.

P1376**Board Number: B823****The TRIP6 LIM domain protein is a positive regulator of the Hippo signaling pathway.**S. Dutta¹, D. McCollum²;¹Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA, ²Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA

The LIM domain proteins have diverse functions, including roles in coordinating cytoskeletal organization with signal transduction and gene expression. A screen in our lab identified TRIP6 as binding partner of the Hippo signaling kinase LATS2. The Hippo signaling pathway is a tumor suppressor pathway that regulates organ size and stem cell fate by maintaining a balance between cell proliferation, differentiation, and cell death. The core pathway consists of the MST1/2 and LATS1/2 kinases and a transcriptional co-activator YAP. When the hippo pathway is inactive, YAP accumulates inside the

nucleus and promotes cell proliferation. When hippo pathway is active, LATS phosphorylates and inactivates YAP. Phosphorylated YAP comes out into the cytoplasm where it is either sequestered or degraded. Other LIM domain proteins such as Ajuba and Zyxin have been shown to be inhibitors of Hippo signaling. In contrast, we find that TRIP6 is a novel activator of Hippo signaling. Depletion of TRIP6 promotes YAP activity by causing decreased LATS-dependent YAP phosphorylation, enhanced YAP nuclear localization, and increased expression of YAP target genes. TRIP6 binds to both LATS2 and MST2 and is required for their activation. Several recent studies have shown that the Hippo pathway is regulated in response to mechanical tension through unknown mechanisms. Interestingly, TRIP6 localizes to cell junctions in a tension dependent manner. TRIP6 also co-localizes with LATS2 at cell junctions implicating it as a potential mediator of the effects of mechanical tension on Hippo pathway signaling.

P1377

Board Number: B824

Human neutrophil Fc gamma receptors activate different cell functions.

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Neutrophils (PMN) are the most abundant leukocytes in the blood. PMN migrate from the circulation to sites of infection, where they are responsible for antimicrobial functions. PMN use phagocytosis, degranulation, and formation of neutrophil extracellular traps (NETs) to kill microbes. PMN also activate genes to produce and release various cytokines. Many of these cellular functions are induced by antigen-antibody complexes engaging specific Fc receptors. However, it is not clear what functions are initiated by a particular Fc gamma receptor. In order to determine what particular neutrophil Fc receptor is capable of inducing phagocytosis, nuclear factor activation, or NET formation, each of the two human Fc gamma receptors was stimulated by specific monoclonal antibodies and these cell responses were evaluated. Fc gamma RIIA crosslinking induced efficient phagocytosis of antibody-coated particles. But it did not induce activation of ERK and Elk-1 in the PMN nucleus, nor it promoted NET formation. In contrast Fc gamma RIIIB crosslinking did not promote phagocytosis, but induced efficient ERK and Elk-1 activation, and also efficient NET formation. Interestingly Fc gamma RIIIB-induced NETs were formed independently of ERK. These data strongly suggest that different Fc gamma receptors promote independent human neutrophil functions.

P1378**Board Number: B825****Modulation of transcriptionally productive BMP4-Smad5 signaling along the endocytic pathway by MxA, a dynamin-family GTPase induced by interferon.**H. Yuan¹, P.B. Sehgal²;¹Cell Biology and Anatomy Department, New York Medical College, Valhalla, NY, ²New York Med Coll, Valhalla, NY

Bone morphogenetic proteins (BMPs) are required for cellular differentiation and mammalian development. Dysfunction of BMP signaling due to mutations in BMPR2 is thought to contribute to pathogenesis of diseases such as idiopathic pulmonary arterial hypertension (IPAH). In humans, PAH is a side-effect of interferon- α (IFN- α) therapy. We investigated the regulation of transcriptionally productive BMP4-Smad5 signaling along the endocytic pathway by MxA, an interferon-inducible dynamin-family GTPase. A BMP4-Smad5-responsive reporter (Id1-Luc) was constructed by using two BMP-response elements from the promoter of the human Id1 gene, a direct BMP4-Smad5-responsive gene. In transient transfection experiments in human HEK293T kidney epithelial cells, this reporter showed a low-level basal level of expression which was induced \sim 100 fold by exposure to BMP4 (10 ng/ml) for 15 hr. The involvement of endocytosis in productive BMP4 transcriptional signaling was confirmed by the observation that cotransfection the reporter construct with dominant negative Dyn2K44A or Epsin2a expression constructs inhibited both basal and inducible Id1-Luc reporter activities. In contrast, overexpression of human MxA markedly enhanced the basal expression by \sim 50-fold, but the maximal inducible levels were unaffected. However, overexpression of MxA could dramatically rescue the Dyn2K44A- or Epsin2a-inhibited basal BMP4 signaling suggesting that MxA increased the basal transcriptional BMP4 signaling probably by enhancing the endocytosis process. We then investigated the ability of MxA to rescue BMP4 signaling in cells overexpressing PAH-disease related BMPR2 mutants (F14 and KDF) which have a dominant-negative effect on BMP4 transcriptional signaling. MxA enhanced signaling from the wt- and KDF-BMPR2 species, both of which reach the plasma membrane. However, MxA could not rescue signaling from the F14 mutant which remains trapped in the endoplasmic-reticulum. Finally, while IFN- α itself inhibited BMP4 signaling, overexpression of MxA rescued this signaling. Taken together, this study links the MxA GTPase to modulation of productive transcriptional signaling elicited by BMP4, and provides evidence for the importance of the endocytic pathway in this process.

P1379**Board Number: B826****Syntaxin4 regulates the surface localization of a promyogenic receptor Cdo thereby promotes myogenic differentiation.**B. Kim¹, M. Ryu², H. Jeong¹, G. Bae², J. Kang³;¹School of Medicine, Sungkyunkwan University, Suwon, Korea, ²College of Pharmacy, SOOKMYUNG WOMEN'S UNIVERSITY, Seoul, Korea, ³Sungkyunkwan Univ Sch Med, Suwon, Kyunggi-do, Korea

Syntaxins are a family of membrane proteins that are involved in docking and fusion of vesicles in exocytosis. Syntaxin 4 is expressed highly in skeletal muscle and regulates the cell surface translocation of the glucose transporter GLUT4. Thereby it regulates insulin-mediated glucose uptake in skeletal muscle which is critical for regulation of whole body insulin resistance. However its role in other processes such as myogenic differentiation has not been investigated. Here we report that overexpression or knockdown of Syntaxin 4 enhances or inhibits myogenic differentiation, respectively. Syntaxin 4 binds to the cytoplasmic tail of a promyogenic cell surface protein Cdo and this interaction seems to be critical for its promyogenic function, including activation of p38MAPK activation and myotube formation. Syntaxin 4 depletion results in a specific decrease in the cell surface resident Cdo without altering another promyogenic membrane protein N-cadherin. Taken together, Syntaxin 4 promotes myogenic differentiation by binding to Cdo and regulating its cell surface translocation.

P1380**Board Number: B827****Reactive oxygen species play a critical role in RANKL-induced osteoclast differentiation.**I. Kang¹, C. Kim²;¹Pharmacology, Inha University, Inchen, Korea, ²Inha University, Incheon, Korea

Osteoclasts are formed by cellular fusions of bone-marrow derived mononuclear/macrophage precursors (BMMs) and are responsible for organic bone resorption. Reactive oxygen species play an important role during osteoclast differentiation. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase transfers an electron from NADPH to molecular oxygen (O₂) to produce superoxide anion (O₂⁻), and its deficiency results in decreased superoxide production in myeloid cells. In this study, we investigated the role of superoxide in osteoclastogenesis, and the target signaling molecules. Treatment of BMMs with antioxidants, such as, superoxide dismutase, *N*-acetylcysteine and diphenylene iodonium significantly inhibited osteoclast formation. This result suggests that NADPH oxidase-derived superoxide may play a critical role in osteoclastogenesis. The membrane subunit of NADPH oxidase, gp91phox deficient mice have defect in superoxide production. The bone volume of gp91phox-null mice was increased compare to wild-type. The osteoclast formation of gp91phox-null mice was decreased to 60% of that of wild-type mice. Moreover, the expression of osteoclast-specific markers, such as, tartrate-

resistant acid phosphatase (TRAP) and cathepsin K were decreased in gp91phox-null osteoclasts, and TRAP activity was also decreased. Nuclear factor of activated T cell 1 (NFATc1) is a master switch for regulating terminal differentiation of osteoclast. Receptor activator of nuclear factor- κ B ligand (RANKL) increased NFATc1 expression, which was decreased in gp91phox-null osteoclasts. In addition, to examine whether oxygen radical can restore the defect in gp91phox-null osteoclasts, we treated gp91phox-null BMMs with H₂O₂, and 0.1 μ M H₂O₂ increased osteoclast numbers to wild-type level. These results suggest that NADPH oxidase-derived superoxide regulates osteoclast differentiation through NFATc1.

P1381

Board Number: B828

Dystrophin is involved to translocation of nuclear factor erythroid 2-related factor 2 under menadione-induced oxidative stress.

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Duchenne muscular dystrophy (DMD) is an X-linked muscular disorder caused by mutations in the dystrophin gene. Although it has been well-known that DMD muscle is more vulnerable to oxidative stress than normal muscle, not much is known about the responsible signal transduction mechanisms. Nuclear factor erythroid 2-related factor 2 (Nrf2) is a transcription factor and regulates the expression of antioxidant enzyme such as Bcl-2, NAD(P)H:quinone oxidoreductase 1 (NQO1) and glutathione S-transferase (GST). In addition, oxidative stress is known to induce translocation of Nrf2 from cytoplasm to nucleus. To study on the responsible signaling pathway, dystrophin knock-downed (dmd) C2 cell line was established by infecting lentivirus particles with dystrophin shRNA. We found that the rearrangement of actin cytoskeleton was impaired when dmd cells were exposed to menadione-induced oxidative stress. To address involvement of Nrf2 under oxidative stress, we examined the expression and translocation of Nrf2 during differentiation of dmd cells. We found that undifferentiated myoblast cells showed no divergence of Nrf2 expression, whereas differentiated myotubes showed that translocation of Nrf2 to nucleus was decreased in dmd cells compared to normal cells under menadione-induced oxidative stress. Moreover, the ChIP assay/PCR analysis showed that the binding of Nrf2 to Bcl-2 antioxidant response element (ARE) was debilitated, as well the transcriptional level of Bcl2 was decreased. These results suggest that the dystrophin is closely linked to translocation and accumulation of Nrf2 to nucleus under oxidative stress.

P1382**Board Number: B829****Retinoic Acid Induces Neuroblastoma Differentiation by Activation of CRABP II.**N. McAndrew¹, G. Gomez²;¹Biology, University of Scranton, Scranton, PA, ²Biology, University of Scranton, Scranton, PA

Retinoic acid (RA) plays a role in cancer cell growth, and is a known chemotherapeutic agent. New evidence has shown the presence of two different proteins in the cell that can directly transport RA to the nucleus: CRABP II shuttles RA to its one nuclear receptor RAR α , triggering differentiation into neuronal phenotypes. In contrast, FABP5 shuttles RA to its other nuclear receptor, PPAR β , and promotes cell proliferation. Studies suggest that the ratio of FABP5 to CRABP II determines which pathway (RAR α or PPAR β) is activated. This ratio is determined either by endogenous expression of these two proteins, or by a RA concentration-dependent activation of the different receptor pathways. To test the latter possibility, SK-N-SH neuroblastoma cells were grown in culture and treated with different concentrations of RA over a four-day time period. RA concentrations varied in increments from 0.01 μ M to 100 μ M by log steps. Cells were subsequently immunostained for RAR α , CRABP II, or FABP5, and relative abundance of each protein was quantified from fluorescence images. Cell growth and differentiation were assessed by cell counts with Trypan Blue stain and quantification of staining density in the nucleus, and then used to determine which receptor, either RAR α or PPAR β , was likely activated. At higher retinoic acid concentrations (1.0-10 μ M), cell differentiation increased. Qualitative immunochemistry showed an increase in RAR α and CRABP II localization in the nucleus, and localization of FABP5 near the cell membrane. At lower concentrations of RA (0.01 μ M -0.1 μ M) FABP5 localized to the nucleus, while CRABP II was localized near the cell membrane. Thus, it appears that the differentiation pathway is determined by RA concentration. These results provide useful insights into neuroblastoma cell differentiation and the potential use for RA as a chemotherapeutic agent.

P1383**Board Number: B830****Effects of a pyrrole-based microtubule poison on RAW264.7 macrophage activity.**J. Ciemniecki¹, C. Lewis¹, J. Gupton², K. Stenger¹;¹Biology, University of Richmond, Richmond, VA, ²Chemistry, University of Richmond, Richmond, VA

The inflammatory response is initiated when macrophages detect a pathogen in the tissues leading to the activation of intracellular signaling pathways. One of these pathways triggers the phosphorylation and subsequent degradation of the I κ B inhibitory protein that is bound to the NF- κ B transcription factor. NF- κ B is then released from its inactive state and translocates into the nucleus, binding to the promoter region of pro-inflammatory genes. This increases the synthesis of several cytokines including tumor necrosis factor- α (TNF- α), as well as the synthesis of enzymes that catalyze the production of reactive molecules. These molecules are integral to destroying the pathogen and recruiting other immune cells to the site of infection. The poly-substituted pyrrole 3,5-dibromo-4-(3,4-dimethoxyphenyl)-1H-pyrrole-2-

carboxylic acid ethyl ester (JG-03-14) is a known microtubule poison and potential anti-tumor agent [i], [ii], [iii]. RAW264.7 macrophages were subjected to treatment with JG-03-14 in order to study its effects on the inflammatory response. After activation with lipopolysaccharide, macrophages exposed to JG-03-14 produced lower levels of intracellular TNF- α and demonstrated a decrease in TNF- α secretion when compared to untreated cells. These cells also showed reduced production of the enzyme inducible nitric oxide synthase resulting in a decrease in the reactive inflammatory molecule, nitric oxide. Further studies indicated that exposure to JG-03-14 decreased the translocation of NF- κ B into the nucleus, which could explain the diminished production of the inflammatory molecules. We conclude that JG-03-14 may act as an anti-inflammatory agent by reducing the production of pro-inflammatory molecules and by altering the secretory pathway of cytokines.

[i] Gupton, J.; Burnham, B.; Krumpe, K.; Du, K.; Sikorski, J.; Warren, A.; Barnes, C.; Hall, I. Synthesis and Cytotoxicity of 2,4- Disubstituted and 2,3,4-Trisubstituted Brominated Pyrroles in Murine and Human Cultured Tumor Cells. Arch. Pharm. Pharm. Med. Chem. 2000, 333, 3–9.

[ii] Mooberry, S. L.; Weiderhold, K. N.; Dakshanamurthy, S.; Hamel, E.; Banner, E. J.; Kharlamova, A.; Hempel, J.; Gupton, J. T.; Brown, M. L. Identification and Characterization of a New Tubulin-Binding Tetrasubstituted Brominated Pyrrole. Mol. Pharmacol. 2007, 72, 132-140.

[iii] Biggers, J. W.; Nguyen, T.; Di, X.; Gupton, J. T.; Henderson, S. C.; Emery, S. M.; Moureq, A.; White, K. L.; Brown, R.; Almenara, J.; Gewirtz, D. A. Autophagy, cell death and sustained senescence arrest in B16/F10 melanoma cells and HCT-116 of 2,4- Disubstituted and 2,3,4-Trisubstituted Brominated Pyrroles in Murine and Human Cultured Tumor Cells. Arch. Pharm. Pharm. Med. Chem. 2000, 333, 3–9.

P1384

Board Number: B831

Estrogen and bisphenols A S regulate lysosomal proteolysis: implications for Lupus.

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Cathepsins are proteases involved in general protein turnover and play important roles in immune function. Specifically, lysosomal cathepsins are responsible for major histocompatibility class II antigen processing. Systemic Lupus Erythematosus (SLE) is an autoimmune disease that is more prevalent in women than in men. The correlation between high estrogen levels and increased severity of the disease implicates this hormone as a regulator of immune function, potentially through binding to the estrogen receptor. Environmental endocrine disruptors such as Bisphenol A (BPA), which are found in many plastic products including food containers, may also be immune regulators. BPA can be absorbed by the foods and liquids that are held in the plastic containers and then can enter the human body. Because of its structural similarity to estrogen, BPA is able to bind to estrogen receptors, which could potentially lead to adverse effects on the body and autoimmune diseases such as SLE. BPA has gained negative

media attention and its removal from products aimed at children was supported by the U.S. FDA in 2013, resulting in its recent replacement in some products by Bisphenol S (BPS). Previous studies in our lab have shown that estrogen and BPA can regulate cathepsin activity in immune cells (B cells and macrophages) and that the regulation varies between control C57Bl/6 mice and lupus-prone NZB/WF1 mice. We now show that cathepsins in non-immune cells are also differentially regulated by physiological and environmental estrogens. To investigate these effects, liver and kidney cells were gathered from lupus-prone NZB/WF1 mice and control C57Bl/6 mice. The cells were treated with either 5 or 50 nM estrogen, BPA, or BPS. The effects of these treatments on cathepsin L activity in liver and kidney cells were analyzed using a fluorescence activity assay. Additionally, to investigate how estrogen may be regulating cathepsins, we examined cathepsin activity in samples pretreated with Fulvestrant, an estrogen receptor antagonist. Our findings show that cathepsin L is being regulated through an estrogen receptor pathway by estrogen and BPA in the liver. Experiments also suggest BPS regulates cathepsin L in the liver. Preliminarily, our results also indicate that estrogen is being regulated through the estrogen receptor pathway in the kidney. We have observed statistically significant differences in cathepsin L activity in liver between mouse strain, type of treatment (estrogen and BPA), and dose of treatment. This suggests that the normal and lupus-prone mice react differently to both physiological and environmental estrogens and that the response is dose-dependent.

P1385

Board Number: B832

Identification and characterization of a new component of the JAK/STAT signaling pathway that regulates cell migration.

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The Janus Kinase and Signal Transducer and Activator of Transcription (JAK/STAT) signaling pathway is involved in essential biological processes including cell fate determination, cell migration, cell proliferation, normal function of immune system, and stem cell maintenance in mammals and fruit fly. We are particularly interested in cell migration since it is not only required for normal embryonic development but can also lead to detrimental outcomes, such as tumor metastasis. Migration of a cluster of cells termed border cells in the *Drosophila* ovary is an excellent example of collective cell migration, which resembles metastasis of some carcinoma cells. Availability of several tools for genetic manipulation and *in vivo* imaging in *Drosophila* make it a suitable model organism to study cell migration. Border cells arise within the follicular epithelium, and are required to migrate to the oocyte to contribute to a fertilizable egg. The requirement for some components of the STAT signaling pathway, including the activating cytokine, its receptor, JAK, STAT and APONTIC, during border cell migration is well-studied, however, the functions of other potential regulators of the pathway during this process are not yet known. To find new components of the pathway that govern cell migration, we knocked down predicted STAT modulators using RNAi expression in follicle cells, and assayed for

defective cell movement. We have shown that BRAHMA (BRM), the *Drosophila* counterpart of yeast SWI/SNF, functions during cell invasion. Target specificity of the RNAi-mediated reduction was verified via rescue experiments by overexpressing the gene in its depleted background. Genetic interaction of *brm* with *stat* is currently being studied through suppression/enhancement assays using different alleles of each gene. To investigate the regulatory effect of BRM on STAT activity further, STAT immunofluorescence intensity in the *brm* mutant egg chambers are being compared to that in wild-type egg chambers. This work could suggest a novel role for BRM in STAT mediated gene expression.

P1386

Board Number: B833

Maintenance of EGFR plasma membrane levels involves transcriptional control of early secretory pathway machinery.

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COPII is the molecular coat machinery that drives vesicle budding from the endoplasmic reticulum. Different COPII coat protein paralogs co-exist in cells, raising the possibility that they form distinct COPII vesicles to transport specific cargo proteins. Interestingly, the activity of the early secretory pathway machinery including COPII is proposed to be regulated by external stimuli such as epidermal growth factor (EGF) [1,2]. Upon EGF stimulation, the EGF-receptor (EGFR) is internalized and degraded by the endo-lysosomal pathway raising the question of how correct plasma membrane levels of EGFR are restored in this situation. Here we show, by applying the Retention Using Selective Hooks (RUSH) system [3], that EGF stimulation specifically increases the transport efficiency of newly-synthesized EGFR from the endoplasmic reticulum to the plasma membrane. This increased transport efficiency is accompanied by transcriptional up-regulation of the inner COPII coat paralogs SEC23B, SEC24B and SEC24D but not of others. These paralogs are required for EGFR transport, as their siRNA-mediated knock-down inhibits EGFR delivery to the plasma membrane. We further show that up-regulation of SEC23B, SEC24B and SEC24D requires the ring finger protein RNF11, which translocates from the cytoplasm to the nucleus upon EGF stimulation. Altogether, our data demonstrate that specific COPII inner coat proteins are transcriptionally up-regulated in response to EGF stimulation. This facilitates the transport of newly-synthesized EGFR from the endoplasmic reticulum to maintain the correct physiological EGFR levels at the plasma membrane after its EGF-induced degradation.

References:

[1] Farhan et al. J Cell Biol. 2010, 189(6): 997-1011

[2] Simpson et al. Nat Cell Biol. 2012, 14(7): 764-774

[3] Boncompain et al. Nat Methods. 2012, 9(5):493-8

P1387**Board Number: B834****Inside the destruction complex: APC's mechanistic role in downregulation of Wnt signaling.**M. Pronobis^{1,2}, N.M. Rusan³, M. Peifer⁴;¹GMB, UNC, Chapel Hill, NC, ²HHMI, Chevy Chase, MD, ³Cell Biology and Physiology Center, NHLBI, NIH, Bethesda, MD, ⁴Department of Biology, University North Carolina, Chapel Hill, NC

Adenomatous polyposis coli (APC), a key negative regulator of Wnt signaling, is mutated in 80% of sporadic colon cancer cases. APC acts in the destruction complex (DC) with the scaffold protein Axin and the kinases GSK3 and CK1 to target β catenin (β cat) to an E3-Ligase for ubiquitination and subsequent proteasomal degradation. Over 20 years of research on the Wnt pathway identified roles for Axin and both kinases. However, APC's function in the destruction complex remains mysterious. Current models generally view the DC as a static protein complex into which β cat is recruited, phosphorylated and then handed over to the E3-Ligase. Axin is thought to have a very slow assembly/disassembly rate in the DC, and increased Axin dynamics has been shown to interfere with β cat destruction, suggesting that formation of a stable complex is key for Wnt signal downregulation. In previous work from our lab, we identified two regions in APC, 20R2 and region B, which appear to negatively regulate association with Axin and are essential for APC's function in the DC. This prompted us to explore the dynamics of APC and Axin in the DC. Our data suggest that the turnover of Axin inside the DC is regulated by binding to APC. We found that APC binds to Axin via two association sites, the well characterized SAMP motifs and a novel Axin-association site that we identified in APC's Arm rpts domain. Both regions are crucial to reduce Axin's dynamics in the DC to form a stable platform for β cat destruction. To investigate this further we used OMX super resolution microscopy and found that changes in the dynamics of Axin caused by APC are associated with changes in the overall structure of the DC. Complexes formed by Axin alone consist of circular Axin cables/sheets in which Axin has a highly dynamic turnover rate. Interestingly, the internal structure of the DC increases in complexity and size in the presence of APC; more Axin cables associate with one another. We propose that APC stabilizes the Axin complex by slowing down Axin's dynamics inside the DC, leading to growth of the Axin scaffold/cables which then increases the overall size of the APC-Axin complex. Besides APC's role in the stabilization of the Axin complex our study also proposes a model of APC's mechanistic function inside the DC. Our data suggest that APC's function in the DC depends on two essential regions, 20R2 and region B. These two adjacent motifs undergo phosphorylation by GSK3 and CK1 and this in turn leads to the disassociation of the novel Axin association site, the Arm rpts, from Axin. Our data suggests that this is a key step in APC's catalytic cycle, facilitating destruction of β cat.

Mechanotransduction 2

P1388

Board Number: B836

Mechanical compression of well-differentiated primary airway epithelial cells causes cellular unjamming and the release of exosomes containing adherens junction proteins.

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Injury of the airway epithelium impairs barrier function and is thought to contribute to asthma pathogenesis. A hallmark of epithelial responses to an injury is initiation of collective cellular migration, but the mechanisms linking injury to migration in airway epithelium has not yet been studied. We have previously shown that mechanical compressive stress (30 cm H₂O) that mimics bronchospasm triggers signals that mediate airway remodeling in asthma. Here, we show that compressive stress also causes stunning changes in collective cellular migration and we propose novel mechanisms that might account for these changes.

Primary human bronchial epithelial cells (HBECs) from normal and asthmatic subjects were grown in air-liquid interface (ALI) culture to differentiate into goblet and ciliated cells. To quantify cellular motion using particle image velocimetry (PIV), we captured time-lapse images of these cells. As metrics of cellular motion we computed cellular mean square displacements (MSD) and we also quantified the lifetime and the size of cooperative cellular clusters or eddies.

Within the first few days of initiating ALI culture (e.g., ALI day3), primary HBECs, whether from non-asthmatic or asthmatic donors, were highly motile and unjammed as indicated by peaks of faster-moving clusters of 10~26 cells. During ALI cultures, the layer of cells became jammed by day 6 in normal cells, while jammed status was not reached until day 14 in asthmatic cells. These results indicate that during differentiation in ALI cultures, the layer of primary HBECs undergoes a phase transition from an unjammed to a jammed state. Compared with cells from normal donors, the transition is delayed in cells from asthmatic donors.

Surprisingly, in well-differentiated and jammed normal cells, mechanical compressive stress drives the jammed phase back to the unjammed phase, as indicated by an over 1000-fold increased in MSD. In uncompressed cells the eddy lifetime could not be quantified within time windows less than 144 minutes, but with mechanical compression eddy lifetime fell to 42 minutes and eddy size was roughly 71 cells. These observations demonstrate a dramatic transition of the well-differentiated epithelial layer from a jammed to an unjammed state. Along with the phase transition, compressive stress induced release of exosomes containing adherens junction proteins, including folliculin. We detected more folliculin in BAL of mice sensitized and challenged with ovalbumin, compared to control mice.

Taken together, our data establish that mechanical compressive stress as expected during bronchospasm cause the monolayer of normal HBECs to transition from a jammed to an unjammed state that is possibly mediated by release of exosomes containing adherens junction prot

P1389

Board Number: B837

Hemodynamic Flow Promotes Mitochondrial Homeostasis in Endothelial Cells.

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The hemodynamic shear flow-induced adaptive responses in vascular endothelial cells have been intensively studied. However, the effects of shear flow on mitochondrial homeostasis in endothelial cells are not clear. We examined the dynamic changes of mitochondrial phenotype and its regulatory proteins associated with mitochondrial functions in endothelial cells under physiological shear stress. Cultured human umbilical vein endothelial cells (HUVECs) were subjected to a constant shear stress (12 dynes/cm²) with a parallel-plate flow chamber system. Mitochondrial biogenesis, dynamics and function were analyzed from ECs after shear treatment. Sheared ECs increased mitochondrial biogenesis as well as a time-dependent increment of mitochondrial fusion revealed by an increase of elongated tubular formation. This phenomenon was coupled with an increased expression of fusion proteins (MFN2, OPA1) but decreased fission protein (FIS1). Consistently, an increase of phosphorylation at S637 but decreased at S616 on Drp1 indicates that shear flow promotes mitochondrial fusion process. Moreover, shear flow increased the expression of mitophagy proteins (PINK1, PARKIN) suggesting that a quality control of mitochondria was enhanced under flow. Shear flow increased the expression of mitochondrial antioxidant enzymes MnSOD2, TRX2, PRX3 and PRX5. As a consequence, an increased mitochondrial membrane potential, ATP production and a decreased intracellular reactive oxygen species (ROS) levels were observed in shear-treated ECs. Our results suggest that hemodynamic flow promotes endothelial function by enhancing mitochondrial homeostasis. Thus, hemodynamic shear flow plays an important role in promoting mitochondrial function in ECs, further substantiates the protective role of shear flow to ECs.

P1390

Board Number: B838

Mechanical compression, Physical Forces and Fluidization in Asthmatic Airway Epithelial Cells.

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In asthma, mechanical compression applied to airway epithelium during bronchoconstriction is thought to trigger a sequence of events that lead to airway remodeling. Initiation and progression of airway remodeling involves epithelial injury-repair processes that require coordinated cellular motions, but the mechanism behind such motions remains obscure. We recently showed that mechanical compression applied to human bronchial epithelial cells causes fluidization of the epithelial layer from a solid-like to a fluid-like phase with associated increase of cell motility and hyper-secretion of the intercellular junction protein folliculin (FLCN) (Park et al. 2014 AJRCCM meeting abstracts). Here we report that this striking phase transition is accompanied by intensified physical forces and altered mechanical cell-cell interaction. To measure local tractions exerted by each cell upon its substrate, and local intercellular forces exerted by each cell upon its immediate neighbors across cell-cell junctions, we used Monolayer Stress microscopy. We discovered that mechanical compression imposed on the epithelial layer causes tractions to increase by 1.2-fold, and intercellular forces to increase by 1.5-fold. Surprisingly, compared to unperturbed normal HBECs, those from the asthmatic airway exhibited 1.3 to 11-fold higher cellular tractions, 1.5 to 5-fold higher intercellular forces, but faster spatial decay of intercellular forces. This demonstrates stronger forces with weaker but more fluid-like cooperativity. To better understand a link between the fluidization and the secretion of FLCN from airway epithelium, we transfected HBECs with short hairpin (sh) RNA targeting FLCN. Remarkably, FLCN deficiency caused tractions to increase by 3-fold, intercellular forces to increase by 2-fold, and collective cell guidance by cooperative mechanical cell-cell interactions to become ablated. Moreover, FLCN deficiency replicated the high mobility observed in compressed HBECs. Finally, the spatial decay of intercellular forces was faster in FLCN shRNA cells, again suggesting a fluidized state with intensified physical forces. Together, these results suggest that mechanical compression mimicking that occurring during bronchoconstriction causes a phase transition of the epithelial layer from a solid-like jammed state to an unjammed fluid-like state, which may be mediated by altered mechanical cell-cell interaction and intensified physical forces.

P1391

Board Number: B839

Modulating primary cilia length restores osteocyte mechanosensing.

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Osteocytes are regarded as mechanosensing cells within bone, potentially using primary cilia as mechanotransducers; yet targeting the primary cilium to modulate osteocyte mechanotransduction is relatively unexplored. Primary cilia are single immotile organelles protruding from the surface of most mammalian cells, and when osteocyte primary cilia are impaired, the cells lose their osteogenic response to fluid flow. A previous study showed that when endothelial primary cilia are elongated, the cells have a greater response to mechanical stimuli. Thus, we hypothesized that increasing osteocyte primary cilia length enhances flow-induced osteogenic gene expression. To increase cilia lengths, MLO-Y4 osteocyte-like cells were treated for 16 hours with 10 μ M fenoldopam (Fen), 500 μ M lithium chloride (LiCl), or vehicle control. Cilia were fluorescently labeled with an antibody against acetylated- α

tubulin, imaged on a confocal microscope, and analyzed with ImageJ. Fen treatment increased cilia lengths by $25.4 \pm 9.1\%$ and LiCl increased cilia lengths by $46.0 \pm 8.9\%$ compared to vehicle control. To examine the response of these cells to mechanical stimulation, cells were seeded onto glass slides, treated with Fen, LiCl, or vehicle control, and exposed to 1 hr oscillatory fluid flow at 1Hz and 1Pa wall shear stress. RNA was isolated immediately after flow and RT-qPCR was used to measure gene expression of osteogenic markers cyclooxygenase-2 (COX-2) and osteopontin (OPN) relative to GAPDH endogenous control. Both Fen and LiCl treatment induced over 50% increases in COX-2 and over 25% increases in OPN expression in response to mechanical stimulation, suggesting that cells with longer cilia are more mechanosensitive. To discern the role of Fen on cilia formation from other potential cellular mechanisms, cilia formation was impaired by siRNA knockdown of IFT88, a gene necessary for proper cilia formation, resulting in a decrease in cilia length and incidence compared to scramble control; and still, Fen treatment was able to restore cilia length and incidence. When exposed to fluid flow, cells with stunted cilia displayed decreased OPN expression compared to scramble control— 7.65 ± 0.58 versus 13.45 ± 0.52 —while Fen rescued this impaired response— 11.59 ± 0.09 . Together, these data suggest that cilia length plays a critical role in cellular mechanotransduction. Furthermore, we have demonstrated that osteocytes with longer cilia are more mechanosensitive, and that this response can be easily modulated by a variety of small molecules. Therefore, increasing cilia length to enhance cellular signaling is a prospective therapeutic strategy that has the potential to rescue malfunctioning cells from ciliopathies that impair primary cilia function.

P1392

Board Number: B840

The vinculin network regulates cytoskeletal remodeling and contractile function in aging myocardium.

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Advanced age is accompanied by significant molecular and cellular remodeling events in the heart which for the past several decades have been thought to contribute to dysfunction. Remodeling of actin-associated proteins, such as those in vinculin/catenin family, has been previously observed in age-related heart failure patients. However, causative mechanisms and functional consequences of remodeling in aged myocardium are still not fully appreciated. Therefore, we examined age-related cytoskeletal remodeling by: 1) assessing conservation of cytoskeletal changes in simian and rat aging

models and 2) examining the functional impact of cytoskeletal remodeling in the rapidly aging and genetically tractable *Drosophila* heart model. High-throughput proteomic quantification of adult and aged simian (11 vs. 22 years) and rat (6 vs. 24 months) left ventricles found significant age-related remodeling of the vinculin network, which was confirmed via histology and western blotting. A screen of wildtype *Drosophila* revealed genotype-dependent vinculin overexpression with age. Cytoskeletal remodeling correlated with cortical stiffening but preserved, not impaired, contractility. We hypothesized that conservation of age-related cytoskeletal remodeling and negative correlation with dysfunction in aging *Drosophila* suggested that vinculin overexpression was a compensatory or beneficial aging response. Therefore, we performed cardiac-specific perturbations of vinculin expression in healthy and myosin-deficient hearts. Vinculin overexpression resulted in cytoskeletal reinforcement, as indicated by increased cortical stiffness, and enhanced fractional shortening and contractile velocities, an index of relative force production, in all adult tissues. Overexpression of vinculin in a myosin-deficient heart was able to rescue cytoskeletal integrity and cellular contractility and partially rescued fractional shortening. To probe a potential mechanism by which the vinculin network could regulate contractility, we performed transmission electron microscopy on sarcomere cross-sections and observed increased myofilament order, as indicated by reduced interfilament spacing variance, in vinculin-overexpressing hearts. Thus, we propose that the vinculin network reinforces the cortical cytoskeleton, resulting in cortical compression, enhanced myofilament organization, and increased contractile efficiency. In sum, these data suggest that cardiac cytoskeleton is a potent regulator of cardiac performance and that cytoskeletal remodeling is a highly-conserved aging event, existing from arthropods to simians, which serves as a compensatory mechanism to maintain mechanical function.

P1393

Board Number: B841

Reading the force map to find centrosome coordinates.

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In 2003 an interesting observation was reported by Burakov and colleagues (Burakov et al, 2003): in vivo, cells maintain actively the centrosome at the cell centre. In a chaotic and constantly changing environment as the cytosol, it is hard to imagine how a specific structure can find its right position and, moreover, how it will keep it. As a wayfarer with a compass, scientists speculate that the centrosome is guided to the right position by microtubules (MTs) (Koonce et al, 1999). Despite scientists agree on the key role of MTs in the coordination of centrosome positioning, nevertheless, it has not been elucidated if MTs respond to a particular signal within and/or outside the cell.

It is increasingly recognised that forces play an important role in the coordination of basilar processes such as polarity establishment, migration, proliferation and differentiation. Forces can derive from the microenvironment and act onto cells, or exerted by the cells themselves onto the surroundings. Forces

are sensed and/or transmitted by dedicated structures named focal adhesions, which represent the anchorage point of actin cables within the cell, and the attachment sites to the extracellular matrix. Changes perceived by focal adhesions trigger signal cascades and lead to actin fibre rearrangements. Here, we speculate that intracellular and extracellular forces resulting in actin remodelling can act as molecular map that will dictate the centrosome coordinates, via MT organisation.

In our set up we prepare polyacrylamide hydrogels that we micropatterned with specific geometries. Definition of a precise geometry let us manipulate cellular force axes. Because the hydrogels are embedded fluorescent nanobeads, we can then calculate cellular forces by measuring beads displacement (experiments of traction force microscopy-TFM). We obtain therefore a complete map of forces within the cell. From the analysis of cellular force map, we noticed the preferential directions of force vectors pointed towards specific coordinates. Furthermore the same position emerged from the analysis of the adhesive site map for the same cells. Curiously, when we stained for the centrosome, we always were able to detect the centriol structures at these coordinates. In conclusion, we propose a new model whereby cellular forces can be interpreted as a map to direct centrosome positioning.

P1394

Board Number: B842

Simultaneous mapping of intra- and extracellular forces at the focal adhesion sites.

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Focal adhesions (FAs) work as mediators of cell-substrate interaction. One of the important read-outs of such an interaction is the traction forces generated at the FAs. The transmission of these forces to the intracellular acto-myosin network is largely facilitated by a complex protein architecture at the FAs. However, how these proteins respond individually to the traction stress is a matter of great interest. Vinculin is a key protein in the regulation and stabilization of FAs. At FA sites, vinculin binds to both actin and talin¹. Hence the tension across vinculin bears the signature of the traction forces at the FAs. We used Förster resonance energy transfer (FRET) based vinculin tension sensor². Briefly, an intramolecular FRET sensor separated by a stretchable peptide linker is incorporated between the vinculin head and tail domains. Therefore the FRET efficiency of the sensor is an effective signature of the tension across vinculin. To measure the traction forces we plated fibroblastic cells on polydimethylsiloxane (PDMS) micropillars³. Such substrates are then used as micro force sensing arrays (μ FSA) wherein deflection of individual micropillar is a read-out for traction forces generated at the corresponding FAs. By combining FRET microscopy with μ FSA techniques, we demonstrate that a simultaneous measurement for both the vinculin tension and the traction forces can be performed at individual FAs. Our results indicate that tension across vinculin exhibit an instantaneous response to traction forces at the corresponding FAs. Furthermore, we used laser ablation technique to control the tension exerted at FA sites. By ablating the stress fibers linked to the FAs we observed an instantaneous decrease of the traction force. The

signature of this perturbation is also seen for vinculin with corresponding increase in the FRET efficiency indicating a strong relaxation of the tension across the molecule. Altogether these data show that the tension across vinculin maps synchronously to the changes in the traction force at the FAs.

References:

1. Ziegler, W. H., Gingras, A. R., Critchley, D. R. & Emsley, J. *Biochem. Soc. Trans.* 36, 235–9 (2008).
2. Grashoff, C. et al. *Nature* 466, 263–266 (2010).
3. Du Roure, O. et al. *Proc. Natl. Acad. Sci. U. S. A.* 102, 2390–5 (2005).

P1395

Board Number: B843

Mechanotransduction via the nuclear lamina.

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How cells respond to physical cues in order to meet and withstand the physical demands of their immediate surroundings has been of great interest for many years, with current efforts focused on mechanisms that transduce signals into diverse phenotypes. Pathways that mechano-regulate the entry of transcription factors into the cell nucleus are emerging, and our most recent studies show that mechanical properties of the nucleus itself are actively controlled in response to the elasticity of the extracellular matrix (ECM) in both mature and developing tissue [1]. The mechano-responsive properties of nuclei are largely determined by the stoichiometry of intermediate filament lamin proteins that line the inside of the nuclear envelope and that also impact upon transcription factor entry and broader epigenetic mechanisms. Signaling pathways are emerging for regulation of lamin levels and cell-fate decisions in response to a combination of ECM mechanics and other molecular cues [2]. Nuclear mechanics also synergize with niche anchorage and cell motility during development, at least in the contexts of adult hematopoiesis [3] and tumor growth [4].

References: [1] Swift et al. *Science* 2013. [2] Buxboim et al *Curr Biol* 2014. [3] Shin et al. *PNAS* 2013. [4] Harada et al *J Cell Biol* 2014.

P1396**Board Number: B844****IN VITRO STUDY OF AGE RELATED HUMAN VASCULAR CELL INTERACTIONS WITH SURFACE MICRO-TOPOGRAPHIES.**H. Chen¹, A. Sales², R. Kemkemer³, A. Greiner¹;¹Zoology, Karlsruhe Institute of Technology, Karlsruhe, Germany, ²New Materials and Biosystems, Max Planck Institute for Intelligent Systems, Stuttgart, Germany, ³Applied Chemistry, Reutlingen University, Reutlingen, Germany

In this work we studied the morphology, migration, and proliferation of primary human endothelial cells (ECs) and smooth muscle cells (SMCs) from donors of different age on differently sized microgrooves made of poly (dimethylsiloxane). We detected cell type-dependent differences in cell morphology but not in cell alignment with respect to the microgroove dimensions. The cell migration of both ECs and SMCs was guided and accelerated by the microgrooves and the proliferation rate of ECs and SMCs was negatively influenced by the microgrooves the deeper the grooves were. While cell morphology and motility on the microgrooves were not influenced by cell age, the proliferation rate of both cell types from old donors was more reduced compared to the cells from young donors. To explain the microgroove-induced reduction of the cell proliferation rate, we investigated the localization of the transcriptional co-activator YAP/TAZ complex. We observed differences in YAP/TAZ localization depending on the dimensions of the microstructure and on the cell age. We elucidated the linkage between environmental physical signals and intracellular responses which influences the cell proliferation. Overall, our work could help to better understand how cell responses to a surface microtopography are dependent on cell type and cell age.

P1397**Board Number: B845****The minimal cadherin-catenin complex forms a catch bond with filamentous actin.**C.D. Buckley¹, J. Tan², B. Pruitt³, B. Weis³, W. Nelson², A.R. Dunn¹;¹Chemical Engineering, Stanford University, Stanford, CA, ²Stanford University, Stanford, CA, ³Stanford Univ Sch Med, Stanford, CA

The linkage between the adherens junction (AJ) and the actin cytoskeleton is important for cell-cell adhesion during tissue development and homeostasis and is often disrupted in diseases such as cancer. Genetic and cell biological studies suggested a model in which the AJ proteins E-cadherin, β -catenin, and the actin binding protein α E-catenin form a minimal cadherin-catenin complex that links cell-cell adhesions to filamentous (F-) actin. Cell culture studies supported this model by showing that E-cadherin is under actomyosin-generated tension that requires α E-catenin, and that E-cadherin-based complexes remodel in response to mechanical stimuli. However, biochemical studies challenged this model by

showing that the minimal cadherin-catenin complex reconstituted from purified components does not bind F-actin stably. Here we reconcile these differences. Using an optical trap-based assay, we find that the minimal cadherin-catenin complex forms stable bonds with F-actin that can survive for several seconds under 5-15 pN of load. Bond dissociation kinetics are explained by a two-state catch bond model in which force shifts the bond from a force-independent, weakly bound state to a force-dependent, strongly bound state. This model may explain how the cadherin-catenin complex binds F-actin and senses mechanical stimuli at cell-cell junctions.

P1398

Board Number: B846

Establishing Mechanical Polarization of the Cell Cortex.

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Polarization plays an important role in a diverse array of physiological processes, including adhesion, migration and cell spreading. Polarized cells spatially regulate protrusive activity, focal adhesion assembly, and traction forces to guide cell migration and tissue morphogenesis. This polarization originates within the actomyosin cortex but the physical and molecular mechanisms are largely unknown. Using micropatterning to control cell shape in conjunction with genetic perturbations to alter the actin cytoskeleton, we identified mechanisms of symmetry breaking within the actin cortex of fibroblasts that establish polarization. We first demonstrated that changes in cell shape promoted a redistribution of traction stresses, but did not alter the total contractile work of the cell. We then identified geometric factors that induced polarization in adherent cells and found that polarization was coincident with the formation of peripheral bundles within the lamella. Hypothesizing that peripheral bundle formation played an important role in mechanical polarization, we sought to identify factors regulating their assembly. Reduced expression of activity of these factors impaired peripheral bundle assembly and, consequently, the onset of cytoskeletal polarization. Our work demonstrates an important mechanical role for peripheral bundle assembly in establishing polarization of adherent cells and creating spatial heterogeneity in the cortex. These processes thus act as self-reinforcing mechanisms through which cells work to actively maintain their shape. In all, these works suggest that the actin cytoskeleton organization could serve as a mechanical mechanism to scale molecular interactions into cellular scale phenomena and polarize cellular activity.

P1399**Board Number: B847****Force Scaling in Stress Fibers.**

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Cells have the remarkable ability to sense geometrical and physical cues from their environment and adapt their architecture accordingly. This process requires a tight regulation of the permanent remodeling of the acto-myosin network, that can both transmit and generate intra-cellular forces. Despite numerous works on the molecular composition of stress fibers, little is known about the mechanism determining the magnitude of force production in these structures. In addition, the relationship between actin cytoskeleton dynamics and intra-cellular force production has yet to be investigated. Here we studied the scaling of contractile force magnitude in stress fibers and investigated the role of actin network dynamics and architecture in this process.

We used micropatterned substrates to control the length and spatial organization of stress fibers in adherent cells and measured the traction forces they produced on deformable substrates. Thereby, we demonstrated that forces scaling exhibit a biphasic behavior. Force magnitude first increased with the length of the stress fibers and then dropped above a critical length. Strikingly, very long cells appeared capable to produce only weak forces. This typical force scaling appeared independent of substrate elasticity and conserved in various cell types having distinct intrinsic cell size limitation.

Monitoring stress fiber relaxation upon laser nano-surgery, we showed that stress fibers were connected to the surrounding actin meshwork all along their length. A theoretical model accounting for the biphasic behavior of force scaling established friction between actin stress fibers and their surrounding cytoskeleton as being a key parameter in the regulation of force production by the cells. It contributed to dissipation of myosin work outside of the fiber. Efficiency of force transmission to focal adhesions appeared regulated by myosin motors distribution and dynamics along the stress fiber, dissipation of stress in adjacent meshwork and connection strength between these stress fibers and focal adhesions. All parameters were further validated experimentally using drugs or actin-binding protein down-regulation.

P1400**Board Number: B848****Protein Tyrosine Kinase AXL and ROR2 Participate in Rigidity Sensing Mechanics.**B. Yang¹, F.M. Hameed¹, Z. Lieu¹, A.D. Bershadsky^{1,2}, M.P. Sheetz^{3,4};¹Mechanobiology Institute, National University of Singapore, Singapore, Singapore, ²Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel, ³Mechanobiology Institute, National University of Singapore, Singapore, NY, ⁴Biological Sciences, Columbia University, New York, NY

Matrix rigidity is an physical cue in the microenvironment that can cause changes in cell morphology, migration, and differentiation. But the mechanism of how cell tests the substrate rigidity is not clear. Recently, submicron pillars study showed that early sensing of substrate rigidity by fibroblasts involves local contractions to a fixed distance of 60 nm irrespective of rigidity (Ghassemi et al., 2012). In a siRNA screen of the human tyrosine kinases, several kinases were found to be required for the cell rigidity sensing (Prager-Khoutorsky et al., 2011). In particular, while control human fibroblasts were less polarized and formed smaller focal adhesions on compliant (5 kPa) substrates than on the rigid ones, the fibroblast lacking receptor tyrosine kinases AXL or ROR2 were shown to have a reduced ability to distinguish between soft and stiff substrate. Using the sub-micron pillar arrays we analyzed the local pillar deflections at an early stage of cell spreading. After AXL knockdown cells produced dramatically larger deflections (pulling distances) than control cells, whereas after ROR2 knockdown cells demonstrated a significantly longer duration of deflections (pulling time). These phenotypic alterations can be rescued by expression of GFP-fused mouse AXL or ROR2 constructs, respectively. In addition, a small molecule inhibitor of AXL, R428, produced the same effect as AXL knockdown, strongly increasing the pillar deflections in the acts of pulling, while the AXL ligand, Gas6, produced the opposite effect. Furthermore, during initial spreading, phospho-AXL and ROR2 were found to localize to the pillar-cell interface. Thus, our studies revealed novel and specific mechanisms for receptor tyrosine kinases AXL and ROR2 involvement in the regulation of cell rigidity sensing.

P1401**Board Number: B849****Optogenetic Spatio-Temporal Control of RhoGEF2 Modifies Cell-Mechanical Activity.**K. Hennig¹, A. Kerjouan², O. Destaing², C. Albiges-Rizo², M. Balland¹;¹Materials, Optics and Instrumental Techniques for the Life Sciences (MOTIV), Laboratory of Interdisciplinary Physics (LiPhy), Grenoble, France, ²Differentiation and Cell Transformation, Institute Albert Bonniot (IAB) Inserm U823, Grenoble, France

Exertion of cell-generated traction forces is crucial for living cells to sense physical properties of their microenvironment. Mechanosensors transduce those mechanical cues into biochemical ones, and vice

versa, by involving various signaling proteins. Here we report an optogenetic approach based on light-induced Cry2-CIBN dimerization to probe to internal dynamics of mechanotransduction. The central regulator protein of acto-myosin cytoskeleton dynamics and cell contractility RhoA was activated by controlling the localization of its upstream activator RhoGEF2. The spatio-temporal control of the biochemical cue of genetically modified REF52s cultured on poly-acrylamide hydrogels was enabled by applying a focalized laser (FRAP-module, 488 nm) at low intensity. Coupling the dynamic biochemical perturbation with traction force microscopy (TFM) at the single cell level allowed examining the cellular force response in terms of contractile energy over time. The light induced diffusion of cytoplasmic RhoGEFCry2mCh to cell membrane located CIBNeGFPcax was observed within seconds and in the range of microns, followed by a dissociation within minutes. Furthermore, subsequent cycles of photoactivation could be triggered. Upon light-induced RhoA activation local stress fiber formation was observed. Counterintuitively, a global decrease in cell contractility was measured during photostimulation. A subsequent regeneration of contractile energy demonstrated the active control of internal forces implying the principle of tensional homeostasis. To conclude, the optogenetic Cry-CIBN system allowed the spatio-temporal control of RhoGEF2 modifying the activity of RhoA leading to a global force response within a rapid temporal scale (seconds). Moreover, the mechanical response in terms of actin reinforcement and phenotype change was dependent on the spatial position of photoactivation and occurred within the minute-range. Hence, it was observed that RhoA mediates cell-mechanical processes which happen at distinct spatial and temporal scales.

P1402

Board Number: B850

Elastic silicone gel substrates control stem cell differentiation, increase growth of brain endothelial cell explants, and promote rapid cancer cell spheroid formation.

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Substrate rigidity impacts cellular migration, gene expression, development, and tumorigenesis, especially in a range of elastic moduli, E , of 0.1 to 100 kPa. Soft substrates commonly used for cell rigidity sensing studies and cell traction force microscopy (TFM) are hydrogels, such as polyacrylamide gels. Elastic moduli of hydrogels can change due to drying, swelling, aging, and hydrolysis of molecular bonds. In contrast, silicone gels are long-term mechanically stable in cell culture media. Here, we formulated a series of mammalian cell-compatible silicone gels with E from a 0.2 to 100 kPa, effectively covering the rigidity range of animal soft tissues. Primary brain endothelial cells (ECs) directly explanted and cultured on 24 kPa silicone gels showed significantly increased growth and better cell-cell contact formations as compared with ECs cultured on plastic dishes. The differentiation of stem cells cultured on the silicone gel substrates had a strong dependence on the substrate rigidity. We also systemically tested the effect of substrate rigidity on multiple cancer cell lines by culturing them on silicone gel substrates with elastic moduli from 0.2 kPa to 96 kPa. Interestingly, we observed that several cell lines,

notably breast (MDA-453) and ovarian (HeLa) tumor lines, consistently form 3D spherical structures on soft gel substrates (

P1403

Board Number: B851

The stretch-activated ion channel Piezo1 transduces matrix mechanics to direct human neural stem cell fate.

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Stem cells integrate multiple internal and external cues in making lineage-choice decisions. Mechanical cues embedded in the environment powerfully modulate stem cell fate. For instance, matrix stiffness strongly influences whether neural stem cells differentiate along a neuronal or glial lineage. The molecular mechanisms linking matrix mechanics to intracellular signaling pathways of differentiation remain unclear. Here we use a multi-disciplinary approach to uncover a new player in mechanosensitive lineage commitment. We find that human neural stem/progenitor cells (hNSPCs) express the stretch-activated ion channel, Piezo1. Mechanical stimulation of hNSPCs elicited a Piezo1-mediated ionic current. In the absence of external mechanical stimulation, cellular traction forces activated Piezo1, resulting in spontaneous calcium transients, which vary with substrate stiffness. Piezo1 knockdown evoked nuclear exclusion of the transcriptional co-activator Yap, suggesting a downstream effector of channel activity. Pharmacological or genetic inhibition of Piezo1 activity reduced neurogenesis and enhanced astrogenesis, demonstrating a role for Piezo1 in neural stem cell fate. We propose that the mechanically-gated ion channel Piezo1 is an important determinant of mechanosensitive lineage choice in neural stem cells and that it may play similar roles in other multipotent stem cells.

P1404

Board Number: B852

EPAC-1 regulates vascular endothelial cell adaptation to fluid shear stress and atheroprotective gene expression.

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Forming the innermost lining of all blood vessels, vascular endothelial cells (VECs) are continuously exposed to the fluid shear stress (FSS) exerted by blood flow. VECs situated in the linear portions of macrovascular structures experience high laminar shear stress (HSS) and adapt by elongating and aligning in the direction of flow. They also display an anti-inflammatory and anti-thrombotic phenotype. At vascular bifurcations and large curvatures, however, FSS dramatically declines. Here, VECs are cobblestone-like in appearance and do not align to flow. Exposure to this low shear stress (LSS) also reduces the expression of numerous vasculoprotective genes, such as Kruppel-like factor 2 (KLF2), endothelial nitric oxide synthase (eNOS) and thrombomodulin (TM), making these areas preferential targets of atherosclerosis development.

Herein, we report a novel role for cAMP-signaling in human aortic endothelial cell (HAEC) adaptation to FSS. Our results showed that cAMP levels increased at flow onset and that inhibition of its synthesis using adenylyl cyclase inhibitor, 2'5'-dideoxyadenosine, reduced elongation and alignment of HAECs cultured under LSS for 48hrs compared to controls. This effect was mediated by cAMP-effector molecule, exchange protein activated by cAMP (EPAC)-1, since EPAC-1 activation improved elongation and alignment compared to controls. Furthermore, knockdown of EPAC-1 and the cAMP-hydrolyzing enzyme, phosphodiesterase (PDE) 4D, abrogated any adaptation to LSS, suggesting that EPAC1 and PDE4D play key roles in shear stress mechanotransduction.

Previously in the lab, we demonstrated that EPAC-1 and PDE4D form a mechanosensory complex with vascular endothelial-cadherin (VECAD) to regulate VEC permeability. VECAD has also been shown to signal with platelet-endothelial cell adhesion molecule (PECAM)-1 and vascular endothelial growth factor receptor (VEGFR)-2 in a mechanosensory complex that facilitates VEC adaptation to flow. Our results show that EPAC-1 and PDE4D knockdown impaired mechanosensory complex signaling by increasing VEGFR-2 phosphorylation and reducing phospho-AKT levels at onset of flow compared to controls, thus abrogating HAEC alignment.

Changes in VEC morphology and cell alignment typically correlate with changes in gene expression. Using quantitative PCR, we demonstrated that flow-mediated increases in KLF2, eNOS and TM mRNA expression were reduced in EPAC-1 and PDE4D knockdown HAECs exposed to LSS, HSS and oscillatory shear stress conditions. Additionally, EPAC-1 knockdown increased leukocyte extravasation across HAEC monolayers *in vivo* and EPAC-1 activation significantly reduced LPS-induced leukocyte rolling flux in mice. Collectively, our data suggests that EPAC-1 plays an important mechanosensory role and may be a potential therapeutic target in atherosclerosis development.

Cytoskeleton–Membrane Interactions

P1405

Board Number: B853

Characterization of higher-ordered septin structures in *Drosophila* cells.

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Septins are highly conserved GTP binding proteins whose deregulation contributes to diseases such as cancer and neurodegenerative disorders. Septins scaffold other proteins and act as diffusion barriers at the plasma membrane and at the midbody during cytokinesis. They also interact with the actin and microtubule cytoskeletons and have been observed to form ordered complexes that can polymerize into higher-order structures such as filaments and rings. The principles of assembly and disassembly of such filaments and rings and their cellular roles are yet to be elucidated. *Drosophila* offers a simple system for studying septins as there are only 5 septin genes: peanut, sep1, sep2, sep4, and sep5 in contrast to 13 septin genes in humans.

We find that 20% of cultured *Drosophila* S2 cells contain tubular structures, up to several microns in length, that label with Peanut immunostaining. These cytoplasmic tubes are present in both mitosis and interphase, suggesting they are not cell-cycle regulated. To investigate the composition and dynamic properties of these higher-ordered structures, we have generated S2 cell lines stably expressing Sep2-GFP, which also localizes to septin tubes. Peanut depletion completely disrupts Sep2-GFP organization, yielding a uniform GFP signal throughout the cell, whereas Sep1 depletion disperses Sep2-GFP tubes into clumps. This indicates that Sep1 and Peanut are required for the formation of the tubular structures and is consistent with previously reported Sep1/Sep2/Peanut complexes. FRAP analysis of Sep2-GFP revealed no recovery over several hours, indicating that the structures are stable and long-lived. However, breakage and re-annealing events were also observed. We have explored the potential relationship between septin tubes and the actin cytoskeleton. Although the tubes did not label for F-actin, treatment with the inhibitor of actin polymerization, Latrunculin A, led to their relatively rapid (

P1406**Board Number: B854****EGF/Ras signaling and the AFF-1 fusogen control seamless tube auto-fusion and shaping in the *C. elegans* excretory system.**F. Soulavie¹, M.V. Sundaram¹;¹Department of Genetics, University of Pennsylvania, Philadelphia, PA

Seamless unicellular tubes are found in the mammalian vasculature and in some invertebrate organs, including the *Caenorhabditis elegans* excretory (renal-like) system. Seamless tubes can form either by hollowing or by cell wrapping followed by auto-fusion. In this work, we are interested in the latter mechanism, which occurs in the *C. elegans* excretory duct. We know that duct cell differentiation is triggered by EGF/Ras signaling. However targets of the EGF/Ras pathway involved in duct differentiation remain unknown. The membrane fusion of the duct cell requires the fusogen *aff-1*. We hypothesize that EGF/Ras signaling upregulates *aff-1* expression to promote duct auto-fusion, and that auto-fusion is important for lumen architecture and function. Here, we used transcriptional reporters to analyze the *aff-1* expression pattern. We already showed that a 5.4kb promoter of *aff-1* is sufficient to promote expression in the differentiating excretory duct cell. Next we will reduce the promoter length to identify which region is required to promote expression in the excretory duct, and test responsiveness of the promoter to EGF/Ras signaling. Next, we used tissue specific expression of *aff-1* cDNA to rescue the auto-fusion of the duct in *aff-1(tm2214)* mutants. First, the same *aff-1* promoter used for transcriptional reporter is able to fully rescue *aff-1(tm2214)* mutants. Second, the *grl-2* promoter, which triggers expression in early excretory duct and pore, rescues duct auto-fusion but is also sufficient to promote ectopic fusion of the duct and pore cells. Finally, the *lin-48* promoter, which is specific to the duct at later stages, is surprisingly unable to promote duct auto-fusion. We demonstrated that only early expression of *aff-1* cDNA in duct is able to rescue duct auto-fusion. Furthermore, these results show a limit in AFF-1 fusogen activity and suggest that AFF-1 may need specific partners or a specific cellular environment in order to promote duct auto-fusion. Furthermore, we investigated the morphological phenotype of the duct cell in these mutants. The duct cell extension is shorter and the lumen also appears thicker and shorter in *aff-1(tm2214)* compared to wild-type. It appears that both excretory duct shape and luminal architecture are altered by loss of duct auto-fusion. These results show that AFF-1 is a key effector of duct tube differentiation. Its early expression specifically in the duct cell makes it a good candidate to be a direct target of the EGF/Ras pathway. By upregulating *aff-1*, the EGF/Ras pathway could promote both seamless-ness and the characteristic elongated duct tube shape. Next we need to confirm *aff-1* as an EGF/Ras target and investigate the relationship between cell fusion and lumen elongation.

P1407**Board Number: B855****Septin filaments recognize micron-scale positive plasma membrane curvature.**A.A. Bridges¹, P. Occhipinti², A.S. Gladfelter²;¹Dartmouth College, Hanover, NH, ²Biological Sciences, Dartmouth College, Hanover, NH

Septins are conserved filament forming GTPases that maintain cell polarity by restricting diffusion of proteins in the plasma membrane and endoplasmic reticulum while acting as a molecular scaffold for cytosolic proteins. Septin higher-order structures form on and associate with the plasma membrane and cells carrying mutated septin genes display irregular cell polarity, abnormal cell shape, are defective at cytokinesis, and are generally inviable. Though it has been shown that Cdc42 drives the accumulation of septins at incipient sites of cell polarity, properties of the plasma membrane that influence and maintain their localization have not been established. Here, we show that septins preferentially bind the plasma membrane in numerous cell types at sites of micron-scale positive curvature, a common topology of polarized cells. In regions of the cell devoid of positive curvature, septin filaments preferentially minimize interacting with negative plasma membrane curvature. Using phospholipid bilayer coated glass microspheres we show that curvature recognition is an intrinsic property of septin filaments. This work demonstrates that septins are a filamentous system capable of sensing plasma membrane shape on the micron-scale. Utilizing this property, septins respond to large scale changes in cell shape and communicate these changes to the cytoplasm.

P1408**Board Number: B856****Furrow ingression drives disassembly of remote microvillar F-actin to liberate membrane for cell surface growth.**L.R. Figard¹, A.M. Sokac²;¹Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, TX, ²Verna and Marrs McLean Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, TX

Morphogenesis is driven by cell shape change, which often requires cell surface growth. Key aspects of this remodeling are still mysterious: What is the source of membrane for cell surface growth? How is membrane transferred to the site of growth? How is membrane transfer regulated? To address these questions, we study *Drosophila* cellularization, a dramatic tissue-building event that expands the embryo's surface area by ~25 fold. We previously showed that microvilli unfold to provide membrane for cleavage furrow ingression during cellularization. We showed that microvillar disassembly is controlled by furrow ingression, and that microvillar membrane slides along the plane of the cell surface into ingressing furrows (Figard et al., 2013). These results provide the first direct evidence for plasma membrane unfolding, which has long been suspected as a broadly conserved mechanism of cell surface growth. But how does furrow ingression drive unfolding and the necessary disassembly of microvillar F-actin cores? We propose that furrow pulling increases plasma membrane tension, antagonizing F-actin

polymerization in microvilli and ultimately causing them to unfold. To validate this proposal, first, we used fluorescence recovery after photobleaching (FRAP) and drug studies to show that microvillar F-actin is constantly polymerizing and depolymerizing. Second, we used 3D time-lapse imaging to show that microvillar F-actin is depleted in sync with furrow ingression. Third, we analyzed a genetic mutant for furrow ingression and found that furrow ingression controls the depletion of microvillar F-actin. Fourth, we used physical force assays to show that furrow ingression exerts a pulling force that is transmitted to far-away microvillar F-actin. In all cells, the surface folds holding the largest membrane stores will likely be supported by F-actin. Our results suggest that plasma membrane tension is a critical regulator of unfolding, acting on F-actin to control the liberation and transfer of membrane during cell surface growth.

P1409

Board Number: B857

Substrate-dependent filament assembly by liver phosphofructokinase-1, the gatekeeper of glycolysis.

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Emerging evidence demonstrates that metabolic enzymes regulating diverse pathways can assemble into filaments that comprise a structural component to eukaryotic cells. We report that phosphofructokinase-1 (PFK1), the first rate-limiting step of glycolysis, assembles into filaments *in vitro* and in cells. We showed by transmission electron microscopy (TEM) and light scattering measurements that *in vitro* filament assembly of PFK-1 is regulated by the substrate fructose 6-phosphate (F6P). In the presence of F6P the liver isoform of PFK1 (PFKL) predominantly formed long filaments measuring up to 250 nm. PFKL filaments are less rigid than actin polymers, displaying right angles in contiguous assemblies. Negative stained EM images showed that filaments are made of stacked tetramers that rotate $\sim 45^\circ$ with each rise. Contacts between PFKL tetramers occur such that the catalytic sites are positioned along the edge of the filament. EM images of PFKL filaments showed that the repeating subunit of the polymer is similar to our recently determined crystal structure of the active tetramer form of PFK1, suggesting that formation of the tetramer precedes formation of the elongated polymer. We confirmed activity-dependent filament assembly with an inactive but tetrameric mutant, PFKL-His199Tyr, that did not form filaments in the presence of F6P, as determined by TEM and light scattering. We next determined PFKL dynamics and filament formation in cells expressing PFKL-GFP. Live-cell confocal microscopy revealed that cytosolic PFKL-GFP was localized to the distal margin of lamellipodia that were devoid of mitochondria where PFKL co-localized with the glycolytic enzymes pyruvate kinase isoform M2 and lactate dehydrogenase A. Live-cell TIRF microscopy revealed that PFKL-GFP formed dynamic punctae. Punctae size was regulated by glucose availability; PFKL-GFP punctae displayed increased size in the presence of the glycolytic inhibitor 2-deoxyglucose. We tested if filament

formation was specific to the PFKL isoform and found that neither the platelet nor the muscle isoforms formed filaments with F6P *in vitro* and in cells. These data indicate that only active PFKL assembles into tetramer-aligned filaments, suggesting filament formation regulates enzyme activity in cells. We hypothesize the activity-dependent recruitment and assembly of PFKL filaments at the plasma membrane provides a scaffolding and structural framework for localized ATP production in lamellipodia which lack mitochondria.

P1410

Board Number: B858

Understanding the underlying mechanisms driving nuclear movement to the periphery in skeletal muscle.

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Nuclei are often schematically represented as a spherical organelle fixed in the center of the cell. This view has greatly changed in the last decades in which nuclear positioning was shown to be essential for vital cellular processes that include cellular division, differentiation and migration. Nuclear dynamicity is particularly active in skeletal muscle where nuclei begin aggregated in the center of the myotube to end dispersed at the periphery of the mature myofiber following a sequence of various types of nuclear movements. We previously reported the underlying mechanisms driving nuclei to aggregate and spread in the center of the myotube to be dependent on microtubules, microtubule-associated proteins (Map7) and motors (kif5b). Furthermore we showed that this process is not dependent of nesprins, nuclear envelope proteins that link the nuclei to the cytoskeleton. We focus here on understanding the later movement of how nuclei move from the center to the periphery of the myofiber.

We developed an *in vitro* system in which we can differentiate myoblasts to fuse into myofibers with peripheral nuclei, aligned sarcomeres and transversal triads. The entire process of nuclear movement of muscle fiber growth can be observed, beginning with nuclei from myoblasts aggregating to the center of the newly formed cell to end with nuclei being anchored at the periphery of the myofibers.

We found that nuclei spread along the center of the myotube before moving to the periphery where they continue to move longitudinally along the fiber. It is only at a later stage that peripheral nuclei become anchored. We demonstrate that peripheral nuclear positioning pre-requires spreading of nuclei along the myofiber, dependent on microtubules, Map7 and Kif5b pathway but independent of nesprins and actin. The movement towards the periphery is however driven by actin and nesprins and is independent of microtubules. We establish that nuclear movement to the periphery of the myofiber is triggered by Amphiphysin 2, a gene mutated in Centronuclear Myopathy (CNM), which recruits and activates N-WASP to regulate actin dynamics. We also show that mutations in amphiphysin-2, associated with CNM, disrupt N-WASP localization and consequentially peripheral nuclear positioning. This work provides evidence for a switch between microtubules and actin-dependent mechanism of

nuclear positioning during myofiber development. Furthermore we establish that nesprins and actin are involved in the movement of nuclei from the center to the periphery prior to anchoring of nuclei at the periphery of the myofiber.

P1411

Board Number: B859

Dynamic remodeling of the airway epithelial cell's contractile machinery in response to cigarette smoke exposure.

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Chronic obstructive pulmonary disease (COPD) is the 3rd leading cause of death in the US with cigarette smoke being the primary insult leading to disease progression. The lung epithelium becomes reactive and undergoes remodeling after exposure to cigarette smoke. The role of cytoskeleton and cell mechanics in the airway response to cigarette smoke is not well understood. We know that cytoskeletal proteins, such as myosin II, actin, alpha-actinin, the catenins, and E-cadherin, are involved in cell-cell adhesion formation and barrier function of the lung. Furthermore, E-cadherin levels decrease in primary epithelial cells from COPD patients. Therefore, we hypothesized that cigarette smoke drives key cytoskeletal protein changes that lead to alterations in barrier function and epithelial repair capacity. To test this, we studied major cytoskeletal (actin and nonmuscle myosin II isoforms) and cell adhesion proteins (E-cadherin) in normal human bronchial epithelial cells (NHBE, collected directly from human patients) or 16HBE cells (primary human bronchial epithelial cell line) grown on an air-liquid interface. Using a Vitrocell smoke chamber, we found that in response to acute smoke exposure (three cigarettes in 36 hours), myosin IIB and actin assembled into apical stress fibers, which are not normally found in airway epithelial cells. Cigarette smoke exposure also resulted in decreased total E-cadherin levels, as well as, decreased E-cadherin at the basolateral surface. Thus, cigarette smoke promotes dramatic acute cytoskeletal changes that likely lead to altered cell mechanical properties (tension, elasticity), which in turn lead to reduced epithelial barrier function. The loss of epithelial barrier function contributes to chronic remodeling leading to COPD progression.

P1412**Board Number: B860****E-cadherin oligomerization regulates the strength of adhesion and collective cell migration.**P. Strale^{1,2}, L. Duchesne³, G. Peyret¹, L. Montel⁴, E. Png⁵, S. Troyanovsky⁶, S. Hénon⁴, B. Ladoux^{1,2}, R. Mege¹;¹Institut Jacques Monod, Université Paris Diderot CNRS, Paris, France, ²Mechanobiology Institute, National University of Singapore, Singapore, Singapore, ³Institut de génétique et développement de Rennes, CNRS, Rennes, France, ⁴Laboratoire Matière et Systèmes Complexes, CNRS, Université Paris Diderot, Paris, France, ⁵Singapore Eye Research Inst, Singapore, Singapore, ⁶Northwestern University, Feinberg School of Medicine, Chicago, IL

Oligomerization of weakly interacting cell adhesion molecules provides the necessary stability and strength to ensure tissue cohesion. Cadherins mediate cell-cell adhesion by forming adhesive bonds between EC1 domains of molecules presented by adjacent cells. Trans interacting cadherins allows the formation of cadherin clusters by a diffusion-trap mechanism further stabilized by anchoring to actin filaments, prefiguring *adherens* junctions found in tissues. Structural and biochemical data show that cadherins also interact in cis via bonds formed between EC1 and EC2 domains of adjacent molecules. These “weak energy” bonds are predicted to shift cadherin clusters from a fluid to an ordered (oligomeric) phase. However, no evidence has been provided so far for the existence of ordered cadherin clusters in vivo and for the cellular impact of cadherin oligomerization. Using nanoparticles to visualize single GFP-tagged proteins in cell membrane at a nanometric resolution, we demonstrate that Ecad molecules arrange in ordered lattices of a few to tens of molecules spaced by less than 10 nm, providing the first demonstration of the existence *in cellulo* of the predicted nanometric arrays of oligomeric cadherin. In contrast Ecad mutated for the two amino-acids responsible for cis-oligomerization display a random distribution. We further studied the consequences of the disruption of the cis interface on cadherin complexes dynamics, *adherens* junction formation, strength of cadherin adhesions and collective behaviour of A431D cells. Bead-cell adhesion assay and electron microscopy analysis shows that the cis interface is not essential for cell adhesion and *adherens* junction formation. Analysis of protein dynamics by FRAP reveal that disruption of the cis interface increases the mobility of junctional Ecad but also of α -catenin as well as of the underlying actin cytoskeleton. Using magnetic tweezers to apply forces on Ecad-Fc coated beads, we demonstrate that this moderate destabilization has a major impact on the stiffness of the anchoring of cadherin clusters to actin. Further analysis of collective cell behaviour in a controlled monolayer expansion assay shows that cis interface disruption increases monolayer fluidity and individual cell migration as a result of decreased cell-cell coordination. Altogether, these data demonstrate that cadherin oligomerization occurs in a cellular context. Unexpectedly, the formation of ordered clusters of Ecad is not required for *adherens* junction formation, but primarily controls the anchoring of the adhesion complexes to the actin cytoskeleton and finally the mechanical stability of cell-cell contacts having a strong impact on collective cell behaviour.

P1413**Board Number: B861****ADENOSINE A_{2B} RECEPTOR INDUCES TRANSITION OF PODOCYTE TO A LESS DIFFERENTIATED STATE.**C. Jaramillo¹, S. Alarcón¹, C. Quezada¹, R.E. San Martín²;¹Bioquímica y Microbiología, Universidad Austral De Chile, Valdivia, Chile, ²Instituto de Bioquímica y Microbiología, Universidad Austral de Chile, Valdivia, Chile

Introduction. It has been described that diabetic glomerulopathy progresses with alterations of the podocyte cells function. The most remarkable feature is the deterioration of the filtration barrier. Interestingly, the nucleoside adenosine increases locally during the progression of diabetic glomerulopathy, probably having a pathogenic role. Our aim will be to determine the role of adenosine in mediating the dysfunction of podocyte cells.

Results. Primary cultured podocytes expressing specific markers were obtained from glomeruli of rats. Exposure of podocytes to adenosine leads to actin redistribution, changes in cellular morphology, decreases of the expression of adhesion molecule integrin $\alpha 3$ and induction of the expression of α -SMA, a marker of epithelial to mesenchymal transition. Further, these changes were accompanied with an increased rate to migration. The above effects were blocked using an antagonist of the adenosine A_{2B} receptor subtype. Moreover, using an antagonist of adenosine A_{2B} receptor *in vivo*, abolished the effacement of foot process of podocytes and the increased levels of proteinuria was blocked in diabetic rats.

Conclusions. Loss of function of podocytes could be mediated by increased levels of adenosine such as in diabetic nephropathy. This effect is relevant because affects the integrity of the glomerular filtration barrier. Furthermore, *in vivo* intervention using an antagonist of adenosine A_{2B} receptor ameliorates the proteinuria in diabetic animals.

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P1414**Board Number: B862****Passive cell membrane regulation in response to mechanical stimuli.**

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Cell processes in any physiological environment ubiquitously involve changes in cell shape, which must be accommodated by their physical envelope – the bilayer membrane. However, the fundamental biophysical laws by which the cell membrane allows for and responds to shape changes remain unclear. Here we show that in response to mechanical stimuli cells rearrange their membrane through a fast passive physical process prior to any active remodeling. Upon application of biaxial stretch, cells recruit membrane area by flattening existing ruffles in lamellipodia. Upon subsequent stretch release, membrane area is stored in the form of tubular invaginations at the cell-substrate interface. In contrast, membrane adaptation in response to changes in medium osmolarity is driven not by area requirements, but rather by the storage of liquid flows expelled by cells. This leads to the formation of the large membrane invaginations in the shape of spherical caps usually described as vacuole-like dilations (VLDs). In both cases, we demonstrate that the shape of the invaginations can be explained by passive minimization of membrane elastic and adhesive energies, constrained by the requirement to store membrane area (in the case of stretch) or volume (in the case of osmotic shocks). Further, we show that by reducing the requirement for adhesion and elastic energy, existing invaginations can act as seeds for additional deformation. By being passive and not depending on specific molecular components, the principles unveiled provide a general mechanism for the adaptation and rearrangement of a significant portion of cell membrane area. Additionally, the local curvature induced by membrane invaginations could potentially trigger downstream mechanotransduction events by affecting curvature-sensitive proteins.

P1415**Board Number: B863****Control of tissue mechanical properties by cellular micro-architecture: cell size, C-cadherin (cdh3) mediated F-actin assembly, and actin bundling.**J.H. Shawky¹, L.A. Davidson^{1,2};¹Bioengineering, University of Pittsburgh, Pittsburgh, PA, ²Developmental Biology, and Computational and Systems Biology, University of Pittsburgh, Pittsburgh, PA

To understand the connection between genetics, the environment, and birth defects we are particularly interested in physical and genetic factors that regulate tissue mechanics in the developing embryo. Tissues within embryos of the aquatic frog *Xenopus laevis* increase in stiffness six-fold as they gastrulate (form the three germ layers) and neurulate (form the central nervous system), however, the origin of this increase is not well understood.

We compare the mechanics of embryonic tissues to the predictions of the cellular solids model (CSM; Gibson, L.J. and M.F. Ashby, Cellular solids: structure and properties. 2nd ed 1997, New York: Cambridge University Press) which has been used to understand the mechanical properties of natural and man-made closed-cell foam materials. The CSM relates bulk stiffness of a foam to the density, or unit-size of individual cells, their microstructural organization, and their material properties. CSM suggested that cell size could be responsible for changes we observe in bulk tissue stiffness. We took advantage of the biology of cell division in *Xenopus* embryos to produce tissues with controlled cell sizes. To generate large cells in tissue explants, we arrested the cell cycle using a combination of cell cycle inhibitors, hydroxyurea and aphidicolin (HUA). Tissues treated with HUA showed a 34% decrease in nuclear density equating to a 50% increase in cell volume. CSM predicts a 23% decrease in tissue stiffness and we found a 25% decrease in the measured elastic modulus. Since cell size plays a role in the large increase in stiffness we sought to test other CSM predictions.

Additional factors contributing to bulk stiffness in closed-cell foams include the thickness and material stiffness of the "cell-wall". To manipulate these factors we sought to reduce F-actin assembly in the cell cortex by expressing a dominant negative C-cadherin (extracellular-domain deleted EP(C)-cadherin; EPΔE) in dorsal tissues. We observed both a reduction in the amounts of cortical F-actin and found dorsal tissues exhibited a 32% decrease in elastic modulus. Efforts are underway to regulate cortical F-actin levels and their degree of cross-linking to test further predictions of the CSM model.

Understanding how cell size, cell cohesion and actin bundling affect tissue stiffness is imperative to uncovering the programs in development responsible for stiffening the embryo.

P1416**Board Number: B864****Elucidating the biomechanics of actin purse-string contraction during epithelial gap closure.**

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Wound healing is crucial to maintain epithelial integrity. Two major mechanisms are widely accepted to regulate epithelial gap closure: cell crawling and actin purse-string contraction. Both mechanisms can coexist and could even influence each other. Here we developed an original in-vitro assay using micro-fabrication techniques, including micro-contact printing, to directly study the closure of model wounds over non-adhesive circular areas. Our observations show that the absence of extracellular matrix is sufficient to exclusively promote an actin-purse string mechanism preventing lamellipodia formation. It appears that the actin cable does not begin as a continuous entity but instead, comes from the assembly of multiple actin cables intermittently anchored to the substrate around the gap edges. This first step is followed by the formation of a continuous contractile cable running across multiple cells. By varying the diameter of the gaps between 100 and 200 μm , we determined a critical length scale over which the forces generated by actin cables contraction remained insufficient in closing the gap. We then determined the influence of cell proliferation and cell-cell junctions in epithelial gap closure. Combining our micropatterning approach with traction force microscopy, we show that the forces exerted by cells on the substrate at the edge of the gap first point away from the center of the gap but then increase in the radial direction pointing towards the gap as closure proceeds. Our results thus provide direct measurements of the forces generated by acto-myosin contraction during epithelial gap closure showing an increase of the radial forces over time and suggesting a possible reinforcement of the acto-myosin cable around the gap. To complete our observations, we performed numerical simulations for which tissues were modeled as visco-elastic materials. These in silico data confirmed the actin reinforcement to explain the dynamic of closure. All together, our experimental data provide the framework for understanding the assembly and the mechanics of contractile actin cables.

P1417**Board Number: B865****Functional coupling between adhesion strength and cell stiffness depends on cytoskeletal tension modulation.**H. Sreenivasappa^{1,2}, S.P. Chaki³, S. Lim^{2,4}, J.P. Trzeciakowski², M.W. Davidson⁵, G.M. Rivera³, A. Trache^{1,2};¹Biomedical Engineering, Texas AM University, College Station, TX, ²Medical Physiology, Texas AM Health Science Center, College Station, TX, ³Veterinary Pathobiology, Texas AM University, College Station, TX, ⁴Chemistry, Texas AM University, College Station, TX, ⁵National High Magnetic Field Laboratory and Department of Biological Science, Florida State University, Tallahassee, FL

Integrin mediated cell-matrix adhesions play a critical role in regulating adaptive cellular responses to stress. Our study investigates the role of cytoskeletal tension on integrin binding strength and cellular remodeling. Adhesion force spectroscopy measurements using a fibronectin functionalized atomic force microscope probe were performed on cells expressing either RhoA or Src variants (wild-type, constitutively active and dominant negative mutants). Cell stiffness at point of contact and adhesion strength of integrin $\alpha 5\beta 1$ to fibronectin were measured. Cell stiffness significantly increased with both RhoA and Src activation. However, the incremental change in cell stiffness was higher in RhoA- than in Src-expressing cells. Consistent with these findings, confocal imaging results showed that RhoA had a significant effect on stress fiber modulation, while Src activation had only a modest effect on actin morphology. The adhesion force of $\alpha 5\beta 1$ integrin to fibronectin also followed a similar trend. The force-stiffness experimental data modeled with a piecewise algorithm, presented a segment showing a flat dependence of force vs. stiffness, while a second segment presented a rapidly increasing positive dependence. The intersection coordinates provided a stiffness threshold corresponding to about 17 kPa, below which the integrin-fibronectin adhesion force is about 37 pN without significant variations. However, above the threshold, the force increases rapidly with the increase in cell stiffness. These results suggest that soft cells present no significant variation in the adhesion force to the matrix until cell stiffness reaches a threshold, after which cells exhibit a significant increase in adhesion strength with increase in cell stiffness, most likely, through a mechanism that involves integrin activation. To further investigate the differential regulation of cytoskeletal tension and cell-matrix adhesions by RhoA and Src, we tested if Src-induced cytoskeletal tension involves modulation of the RhoA pathway. We used SU6656, a specific Src kinase inhibitor, in cells expressing RhoA constitutively active mutant to test mechano-sensitive response of cells to Src inhibition. SU6656 treatment suppressed the increase in both cell stiffness and adhesion strength induced by RhoA activation, by releasing RhoA-induced contractile tension. Moreover, western blots showed decreased Src and myosin phosphorylation due to SU6656 inhibition. Taken together, these results suggest that Src modulates the RhoA pathway through regulation of myosin activation, which is a downstream effector of RhoA.

P1418**Board Number: B866****Cytoskeletal forces during T cell activation.**K. Hui¹, A. Upadhyaya²;¹Physics, Institute of Physical Science and Technology, College Park, MD, ²Physics and IPST, University of Maryland, College Park, MD

T cell activation is critical for the adaptive immune response in the body. The binding of the T cell receptor (TCR) with antigen on the surface of antigen presenting cells (APC) triggers signaling cascades and cell spreading. Physical forces exerted through the TCR have been shown to induce signaling events, but the origin of how these forces are generated and maintained is unknown. Here, we use traction force microscopy to measure the forces exerted by Jurkat T cells during TCR activation. We used anti-CD3 coated elastic polyacrylamide gels to stimulate Jurkat T cells and measured the spatially resolved traction stress map exerted by these cells as they were activated. Perturbation experiments revealed that stresses were largely generated by actin assembly and disassembly and regulated by the flow speed of actin. Our experiments further suggest that TCRs are structurally linked to the actin cytoskeleton through the Arp2/3 complex. On the other hand, we found that myosin II motor activity was dispensable for maintenance of traction stresses, but was important for traction stress generation. Finally, we investigated calcium influx in Jurkat T cells when activated on substrates of physiologically relevant stiffnesses. Our results highlight the importance of cytoskeletal forces for receptor activation in T cells.

P1419**Board Number: B867****Formation of transcellular tunnels by mechanical force.**W. Ng^{1,2}, K.D. Webster^{2,3}, C. Stefani⁴, E. Lemichez⁴, P. Bassereau⁵, D.A. Fletcher^{3,6,7};¹Graduate Program in Bioengineering, University of California, Berkeley, Berkeley, CA, ²Bioengineering, University of California, Berkeley, Berkeley, CA, ³Biophysics Graduate Group, University of California, Berkeley, Berkeley, CA, ⁴INSERM, Nice, France, ⁵PhysicoChimie Curie, Institut Curie, Paris, France, ⁶Department of Bioengineering, University of California, Berkeley, Berkeley, CA, ⁷Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA

The endothelial monolayer in blood vessels serves as a physical barrier that separates the underlying tissue from the bloodstream. A group of bacterial toxins comprising EDIN or C3, two ADP-ribosyltransferases targeting RhoA, and several adenylate cyclases disrupt this protective barrier by inducing the formation of transcellular tunnels through fusion of the dorsal and ventral plasma membrane of a cell. These transcellular tunnels enlarge by a physical phenomenon of cellular dewetting. However, the mechanism of the initial fusion event during tunnel formation remains poorly understood. All these toxins reduce actomyosin contractility either by inactivating RhoA directly or by inducing a cAMP/EPAC/Rap pathway. Relaxation of the cytoskeleton is believed to increase the likelihood that the dorsal and ventral membranes of the cell come into contact and fuse to form a transcellular tunnel. To

test whether a tunnel will form when membranes are brought into close apposition, we used atomic force microscopy (AFM) to apply a localized mechanical force and combined this technique with total internal reflection fluorescence (TIRF) microscopy to visualize the formation of transcellular tunnel formation. We found that mechanical forces are sufficient to induce the formation of transcellular tunnels in HUVECs in the absence of toxin, and we observed localization of the I-BAR domain of MIM and actin around the edges of the transcellular tunnel, similar to toxin-induced tunnels. We also found that cells exposed to the EDIN/C3 toxin formed transcellular tunnels at lower forces than cells not exposed to the toxin. Our study suggests that the cytoskeleton provides resistance against spontaneous formation of transcellular tunnels, a mechanism that could be hijacked by bacterial pathogens to breach the protective endothelial barrier.

P1420

Board Number: B868

A novel frameshift mutation in caveolin-1 associated with pulmonary arterial hypertension down-regulates caveolin-1 levels, reduces caveolae stability, and decreases the buffering capacity of caveolae.

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Caveolin-1 is a major structural component of caveolae, flask-shaped invaginations of the plasma membrane found in multiple cell types. Recently, C-terminal frameshift mutations in the CAV1 gene have been identified in patients with pulmonary arterial hypertension (PAH), a lethal disease of the pulmonary microvasculature (Austin et al. *Circ Cardiovasc Genet.* 2012;5:336-343.) These findings suggest that defects in caveolae may contribute to the pathogenesis of PAH. To test this idea, in the current study we probed for defects in caveolae in human skin fibroblasts obtained from PAH patients heterozygous for mutant CAV1. We found that levels of caveolin-1 and some of its known accessory proteins such as caveolin-2, cavin-1 and EHD2 were significantly decreased in patient fibroblasts compared to controls as assessed by Western blotting. Peptides from the new C-terminus of mutant CAV1 were identified by mass spectrometry from patient cell lysates, suggesting the down-regulation of caveolin-1 is not simply due to haploinsufficiency. Despite the decreased levels of caveolae-associated proteins, intact caveolae could be detected by electron microscopy and biochemical fractionation in patient fibroblasts, and immunofluorescence microscopy revealed that both caveolin-1 and its accessory proteins colocalized normally in caveolae. However, the stability of caveolin-1 complexes and caveolae were decreased in patient fibroblasts compared to controls, implying that the presence of the mutant protein impacts caveolae stability. Furthermore, the viability of patient's cells significantly decreased under hypoosmotic stress compared to controls, indicating that the decreased of number and/or stability of caveolae impair the ability of caveolae to function as a membrane reservoir. These findings suggest that the decreased levels of caveolin-1 and reduced stability of caveolae resulting from expression of mutant CAV1 may both contribute to the development of PAH.

P1421**Board Number: B869****Confocal microscopy and solution NMR studies of cytoskeletal proteins from *Tetrahymena thermophila*.**R.C. Sterner¹, A.M. Kilpatrick², J. Honts³;¹Biology, Drake University, Des Moines, IA, ²Physics, Drake University, Des Moines, IA, ³Drake University, Des Moines, IA

Organisms derive their complexity from the associations of cells to form tissues with unique structures and functions. Although the fresh water ciliated protist *Tetrahymena thermophila* is only a single cell, it demonstrates remarkable complexity in its cortical cytoskeleton. From cell to cell, this intricate cell surface architecture must be faithfully replicated. The molecular mechanisms of its organization and regulation during division are not completely documented. In previous studies, we have identified nearly three dozen proteins through proteomic analysis of calcium-dependent contractile fibers isolated from the cortical cytoskeleton of *Tetrahymena thermophila*. Proteins investigated include a putative calcium-binding protein (Tcb2), a filament-forming structural protein (Epc1), and a protein kinase (Epk1), among others. These proteins have been hypothesized to function in the calcium-dependent regulation of ciliary motion and are believed to play key regulatory and structural roles. Confocal laser scanning microscopy as well as solution NMR have been employed in order to further investigate these dynamic protein interactions over the course of the cell division cycle to form the complex scaffold of protein fibers and filaments and to better comprehend their structure-function relationship. Through the use of confocal microscopy, we have found that these protein localizations are present in only a narrow layer in the cells cortical cytoskeleton, specifically the epiplasm. This narrow epiplasmic distribution is consistent with the purification results we have seen from the membrane skeleton of this cell. In addition, we have reconstituted calcium triggered contractile fabrics with Tcb2 *in vitro*. The C-terminal domain of Tcb2 is highly soluble and amenable to structural characterization while full-length Tcb2 forms filamentous structures and becomes insoluble in the presence of calcium and/or upon concentration. NMR spectra indicate that the C-terminal of Tcb2 is well folded in the presence and absence of calcium, and upon calcium addition, a dramatic conformational change occurs. In order to quantify the calcium binding properties of this domain, we are utilizing NMR spectroscopy. In the future, Tcb2 interactions with other cortical cytoskeleton proteins such as Epk1 and subdomains of Epc1 will be explored through NMR experiments. The distribution of these cytoskeleton proteins relative to one another will be investigated through both FRET and double labeling studies.

P1422**Board Number: B870****Intracellular crystallization of insect virus and polyhedra revealed by quick-freeze, freeze-fracture electron microscopy.**N.i. Morone¹, H. Mori², J.E. Heuser¹;¹iCeMS, Kyoto University, Kyoto, Japan, ²Kyoto Institute of Technology, Kyoto, Japan

Natural silkworm (*Bombyx mori*) as lepidopteran larvae can be infected by cytoplasmic polyhedrosis viruses (Cypoviruses) of icosahedral ds-RNA viruses. The cypoviruses quickly become embedded in elaborate protein crystals called "polyhedra". X-ray diffraction about the internal crystal structure of these purified polyhedra has been succeeded (Coulibaly et al., 2007), but the molecular mechanism by which they assemble and incorporate virions in the cytoplasm of infected insect cells remains obscure. Here, we have examined their molecular mechanisms of assembly in their natural environment inside intestinal and silk gland cells of larval silkworms, and in Sf21 cells in tissue culture. Avoiding the artifacts and difficulties of previous EM-approaches, all specimens were here prepared by "quick-freezing" directly from life, followed by freeze-fracture to gain entry into the cells, and then "deep-etching" and rotary Pt-replication to achieve true-to-life, topologically accurate 3-D images of the polyhedra and their associated virions. This approach yielded novel and definitive images of polyhedra crystallization in situ, and thereby revealed unique differentiations within cellular environs wherein polyhedra organize, and the exact mechanism of inclusion of nascent virions into the polyhedra. Specifically, it allowed us to focus directly on the edges of polyhedra, right where the nascent viruses are being captured and engulfed during the growth of the polyhedra. This allowed us to compare the success of virion-capture and polyhedron-assembly in a variety of different experimental conditions, including the substitution of mutant viruses and incomplete virions lacking critical recognition-factors. Additionally, it allowed us to determine exactly how the cytoskeletal architecture of the infected cells is altered, to permit this elaborate crystal-formation and virus-incorporation to occur. Especially, we found the crystal formation in the silk gland cell with cypovirus-free system, which we have succeeded in the preparation. In this presentation, our observations and results will be described and illustrated by means of realistic three-dimensional images presented in "anaglyph" stereo form.

P1423**Board Number: B871****Tcb2: a novel *Tetrahymena* calcium-binding protein that is part of a contractile assembly.**

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Tetrahymena thermophila, a ciliated protozoan, exhibits a complex cell surface architecture, organized by a membrane-associated cytoskeleton. Two specific proteins, Epc1, a coiled coil protein, and Tcb2, an EF-hand calcium-binding protein were major components of a contractile gel that formed when an alkaline extract of the *Tetrahymena* membrane skeleton was adjusted to neutral pH in the presence of calcium ions. These two proteins co-assembled to form cross-linked filament networks. To study Tcb2 in detail, we engineered genes for optimized expression of Tcb2 protein, as well as its N-terminal and C-terminal domains. Purified full length Tcb2 protein (a) exhibited a clear calcium-dependent shift in electrophoretic mobility in native polyacrylamide gels, and (b) formed fibrous aggregates when calcium ions were added. Electron microscopy revealed the presence of globules and short filaments. Steady-state fluorescence spectroscopy was used to monitor conformational changes in the Tcb2 C-terminal domain (Tcb2-C) during titration with calcium ions. This indicated the presence of one high affinity calcium-binding site, with a low micromolar dissociation constant. The affinity of Tcb2-C for calcium falls within the range of calcium concentrations shown to trigger specific biological functions within the ciliate cortex. The shift of peaks observed during calcium titrations of Tcb2-C monitored by (1)H-(15)N HSQC-NMR provided complementary evidence for calcium-dependent conformational changes. We have obtained protein crystals of Tcb2-C that diffract to $\leq 2.7 \text{ \AA}$, and work is underway to determine the atomic structure of this domain. The N-terminal domain (Tcb2-N) has demonstrated very different properties, especially aggregation in the presence of calcium ions, which may explain why the intact protein readily formed filamentous aggregates in vitro.

Integrins and Cell–ECM Interactions 1

P1424

Board Number: B873

Comparison of the effect of the vinexin family proteins on vinculin-mediated mechanosensing.

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The vinexin family protein, also called the Sorbs protein, is composed of one sorbin homology (SoHo) domain and three SH3 domains. This family is conserved among multicellular animals and includes vinexin (or Sorbs3), CAP (or Sorbs1) and ArgBP2 (Sorbs2). They are localized at focal adhesion (FA) sites or cell-cell adhesion sites, and have common binding proteins including cytoskeletal molecules like vinculin, as well as signaling molecules such as c-Abl and c-Cbl. However, they have different functions in vivo; vinexin is involved in wound healing, CAP regulates insulin signaling and ArgBP2 functions as a tumor suppressor. Recently we reported that the interaction of vinculin with vinexin plays a key role in sensing extracellular matrix (ECM) stiffness. In addition, Bharadwaj R. et al. reported that Sorbs protein in *Drosophila* regulates assembly and function of two tension sensing organs and Martin M. et al. reported that ArgBP2 maintains endothelial contractility and vessel lumen integrity. These emerging reports suggest that Sorbs proteins work as a ‘mechanosensor’. Because cells usually express one or more Sorbs proteins, it is difficult to compare the functions of Sorbs proteins exactly. In this study, we established Sorbs-triple depleted mouse embryonic fibroblast cells (referred to TKO MEF), and then compare the functions of Sorbs proteins using the cells re-expressing each Sorbs protein. We first analyzed the localization of Sorbs protein in TKO MEF. Immunostaining revealed that vinexin and CAP were co-localized with vinculin at FAs, while ArgBP2 was co-localized with alpha-actinin on actin stress fibers. We also found the area of FAs defined by vinculin was increased in each Sorbs protein re-expressing cells. Previous our results that vinexin is required for ECM stiffness-dependent behaviors of vinculin prompted us to examine the effect of Sorbs protein on vinculin status. Interestingly, both vinexin and CAP significantly increased the Triton X-100 insoluble vinculin on rigid ECM compared to on soft ECM, whereas ArgBP2 did not. Fluorescent recovery after photobleaching (FRAP) analysis revealed that both vinexin and CAP significantly increased the immobile fraction of vinculin on rigid ECM, whereas ArgBP2 did not. These results suggest that both vinexin and CAP, but not ArgBP2, are required for ECM stiffness-dependent stabilization of vinculin at FAs. To uncover the mechanism of these differences, we investigate the interaction of Sorbs proteins with vinculin using purified protein. Pull-down experiment showed that both vinexin and CAP strongly interact with vinculin but ArgBP2 hardly. Taken together, in MEFs, both vinexin and CAP, but not ArgBP2, interact with vinculin and contribute to vinculin-mediated mechanosensing. This is a first report of the comparison of Sorbs proteins using cell lines expressing a single Sorbs protein and purified Sorbs proteins.

P1425**Board Number: B874****Desmoplastic 3D-adhesion structures orchestrate a tumor-associated ECM induced myofibroblastic phenotype.**J. Franco-Barraza¹, T. Luong¹, N. Shah¹, R. Madhani¹, G. Cukierman², K. Alpaugh¹, K. Devarajan¹, J. Hoffman¹, R. Uzzo¹, E. Dulaimi¹, E. Cukierman¹;¹Fox Chase Cancer Center, Philadelphia, PA, ²University of North Carolina, Chapel Hill, NC

The desmoplastic microenvironment plays a pivotal role in tumor development/progression. This fibrotic niche is produced by myofibroblastic cells and is commonly observed in tumors such as pancreatic adenocarcinoma (PDAC) and renal cell carcinoma (RCC). We have shown that quiescent fibroblasts become activated, undergoing a desmoplastic (i.e., myofibroblastic) phenotypic switch triggered by tumor-associated (TA) but not by normal fibroblast-derived extracellular matrix (ECM). However, the molecular mechanisms responsible for this process remain unclear.

Using syngeneic human fibroblasts harvested from patient-matched normal and tumor samples, we produced a human mimetic pancreatic and renal 3D stromal system to study the role of myofibroblastic activator TGF β and ECM integrin receptors $\alpha_v\beta_5$ and $\alpha_5\beta_1$ in the TA-ECM-induced phenotypic switch. We assessed the role of known integrin downstream effectors, non-receptor tyrosine kinases FAK and Src, and tested their roles in the observed TA-ECM-induced phenotypic switch. Approaches included the use of specific inhibitor and activator drugs as well as genetic manipulations. Our data suggest that while TGF β is indeed needed for the production of the particular TA-ECM, it is no longer required during the matrix induction of the phenotypic switch. Results suggest a cross-talk between integrins $\alpha_v\beta_5$ and $\alpha_5\beta_1$ necessary for maintaining the phenotypic switch.

We uncovered a mechanism whereby $\alpha_v\beta_5$ -integrin activity, in a Src/FAK-dependent manner, triggers the redistribution of FAK-independent $\alpha_5\beta_1$ -integrin activity away from desmoplastic 3D-adhesion structures. This in turn prevents this integrin from inhibiting the TA-ECM-dependent phenotypic switch. To validate the *in vitro* results, we used a multi-fluorescent labeling approach to simultaneously detect tumoral vs. stromal compartments and question the localization and activity levels of $\alpha_5\beta_1$ -integrin in the original pathological tissue samples. Finally to establish clinical relevance, the same approach was combined with a novel computational code applied, as a batch analysis, on a large human renal and pancreatic tissue cohort.

We conclude that outcome-predictive and TGF β -dependent matrix-induced stroma activation is maintained by Src/FAK-dependent $\alpha_v\beta_5$ -integrin activity. This activity triggers the exclusion of myofibroblastic-inhibitory and FAK-independent $\alpha_5\beta_1$ -integrin activity from altered 3D-adhesions. We propose that this desmoplastic mechanism may not only comprise a clinically important occurrence but also a new bio-marker to assess stromal index in cancer patients.

P1426**Board Number: B875****Pro-Leu-Gly hydroxamate inhibited adhesion and differentiation of CD133+CD44+ prostate cancer stem cells via Rho associated Kinase1 (ROCK1) and Focal Adhesion Kinase (FAK) pathway.**S. Rentala¹;¹GITAM University, Department of Biotechnology (GIT), Visakhapatnam, India

Prostate cancer has become a global health concern and is one of the leading causes of cancer death of men after lung and gastric cancers. In this study, we examined whether Pro-Leu-Gly hydroxamate, an inhibitor of Matrix metalloproteinases (MMPs) has any effect on adhesion and differentiation of prostate cancer stem cells. To this end, we analyzed the influence of Pro-Leu-Gly hydroxamate on the cell adhesion and differentiation of CD133+CD44+ cells derived from prostate cancer biopsies and peripheral blood. CD133+CD44+ cells were treated with Pro-Leu-Gly hydroxamate (20 - 50 μ M) for different time periods. Cell adhesion to endothelial cell monolayers and differentiation into prostate cancer cells were evaluated. α 1, β 1 and α 2 β 1 integrins adhesion receptors and the Rho-dependent kinase (ROCK) and focal adhesion kinase (FAK) were analyzed by Western blot. Further blocking studies with the ROCK inhibitor H1152, anti-FAK antibody and anti-integrin α 1 and β 1 antibodies were carried out. Pro-Leu-Gly hydroxamate treatment inhibited dose-dependently cell attachment to endothelium and differentiation. The inhibitory effect of Pro-Leu-Gly hydroxamate on cell adhesion was associated with decreased expression of integrins α 1 and β 1 and phosphorylated MYPT1 and FAK. Furthermore, Pro-Leu-Gly hydroxamate strongly reduced ROCK1 and FAK mediated differentiation of CD133+CD44+ cells, which was confirmed by antibody treatment. Pro-Leu-Gly hydroxamate modified the expression of cell adhesion molecules and differentiation markers. These beneficial effects of Pro-Leu-Gly hydroxamate may be mediated by ROCK and FAK signaling pathway. The data presented may point to novel treatment options for prostate cancer

P1427**Board Number: B876****Characterization of the mechanotransduction in response to the secreted protein Netrin-1.**K. Uriot¹, P. Girard¹, M. Coppey-Moisan¹, O. Faklaris¹, F. Lam¹, O. Blanc¹;¹Institut Jacques Monod, Paris, France

Netrin-1 is a secreted protein known to attract neurons expressing the membrane receptor DCC (Deleted in colorectal cancer) during nervous system development. However, Netrin-1 is present in many tissues where it interacts with plasma membranes and proteins of the extracellular matrix such as heparin. Thus, it can modulate cell adhesion and migration and notably in colorectal epithelia where Netrin-1 is secreted at the basis of the crypt and DCC is present at the surface of the villi where they

have been implicated in carcinogenesis. Moreover, Netrin-1 has been shown to interact with integrins alpha6beta4 and alpha3beta1. Since DCC as well as integrins can directly bind to FAK (Focal Adhesion Kinase ; known for its role in mechanical responses) and potentially lead to its phosphorylation, we propose to decipher the molecular mechanisms leading to Netrin-1-induced mechanotransduction by the mean of different mutants. At the cell level, we evaluate the ability of the protein to diffuse toward and to bind to its receptors in colorectal cell lines or in primary neurons. At the single molecule level, we mimic the immobilization of Netrin-1 on coated substrates and we observe how certain domain deletions impact on DCC dynamics in the plasma membrane when we control the interaction Netrin-1/integrins and Netrin-1/DCC. To do so, we use superresolution microscopy in 3D (PALM/dSTORM) or in live (sptPALM). Moreover, we evaluate the importance of integrins and other force-transducing partners such as FAK or actin in DCC-expressing cells in order to characterize how mechanotransduction is involved in Netrin-1 signaling in cancer and during development.

P1428

Board Number: B877

miR-625# and miR-206 participate to the cellular response to matrix rigidity.

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The extracellular matrix (ECM) affects many aspects of cell growth and behavior. Not only do cells respond to the composition of the ECM, but they also respond to its physical properties. In a seminal paper, Pelham and Wang observed that cells develop large integrin-based adhesions on rigid surface, whereas on soft substrate adhesions remain immature and small. Since this cellular response to ECM stiffness is central in various aspects of biology, from physiology to disease, many efforts have been directed recently to understand how cells “sense” and respond to the stiffness of their environment. However this remains, so far, an open question. In this study, we investigated the role of micro RNAs (miRNAs) in the cellular response to matrix rigidity. Comparing miRNAs expression in fibroblast cultured on soft (1kPa) and stiff substrates (25kPa), we observed that ECM rigidity induced a decrease in miR-625# and miR-206 expression. Interestingly, inhibition of myosin II increased miR-625# and miR-206 expression, indicating that cell-generated tension regulates miR-206 and miR-625# expression. Previous work have shown that fibronectin is a direct target of miR-206, consistent with this we found that miR-206 expression in cells cultured on stiff substrates decreased fibronectin expression. Additionally, we found that miR-625# expression decreased cell spreading and survival on rigid ECM. These data indicate that miR-206 and miR-625# regulate the cellular response to ECM rigidity. We are currently pursuing this work to identify miR-625# targets which impact cell adhesion and survival.

P1429**Board Number: B878****Compliance-dependent Extracellular Matrix Remodeling in Salivary Gland Morphogenesis.**S.B. Peters¹, D.A. Nelson¹, M. Larsen¹;¹Biological Science, University at Albany, SUNY, Albany, NY

Mechanical properties of the microenvironment regulate cell morphology and differentiation within complex organs. However, the mechanisms through which mechanical signals are translated within a tissue are poorly understood. Using mouse submandibular salivary gland organ explants that are mechanically sensitive, we investigated whether alterations in the extracellular matrix and basement membrane occur as the organ responds to changes in extracellular compliance. We examined the assembled, DOC-insoluble extracellular matrix (ECM) in organ explants that were grown on polyacrylamide gels having high compliance (similar to in vivo embryonic conditions), low compliance (i.e. high stiffness, similar to pathologic conditions) and in explants that were grown at low compliance for 72 hours and then transferred to high compliance gels for 24 hours, which we referred to as “mechanical rescue.” ECM assembly was disrupted in the glands grown at low compliance compared to those grown at high compliance, which was evident by thinner BM around the proacini. Incomplete clefts, indicative of defective morphogenesis, were also detected in glands at low compliance, which correlated with a decrease in the myoepithelial cell marker protein, smooth muscle α -actin. Mechanically rescued glands demonstrated a substantial recovery of the basement membrane and cleft progression, together with an increase in myoepithelial cell differentiation. We detected a basal accumulation of active integrin b1 in the differentiating myoepithelial cells that formed a continuous peripheral localization around the proacini, as well as localizing in clefts within active sites of morphogenesis, in explants that were grown at high compliance or that were mechanically rescued. This activation of integrin b1 in clefts and adjacent to the basement membrane in proacini was interrupted in explants grown at low compliance. Exogenous TGFb1 delivered to glands grown at low compliance stimulated ECM and basement membrane assembly and localization of active integrin b1 in the myoepithelial cells, while inhibition of TGFb receptor signaling with SB431542 during mechanical rescue prevented restoration of ECM/basement membrane, myoepithelial cell differentiation, and localized integrin b1 activation. These results indicate that ECM and basement membrane assembly and myoepithelial differentiation are compliance-dependent during salivary gland development. Further, our results indicate that TGFb-signaling is required for this compliance-dependent tissue remodeling, which may require integrin b1 activation. Supported by NIH/NIDCR R21DE02184101 and R01DE022467.

P1430**Board Number: B879****Role of the fibronectin synergy site in modulating integrin recruitment and molecular-scale force generation.**A.C. Chang¹, A.H. Mekhdjian¹, M. Morimatsu¹, A.R. Dunn¹;¹Chemical Engineering, Stanford University, Stanford, CA

Integrins are heterodimeric transmembrane adhesion receptors that connect the cell to the extracellular matrix, and play central roles in transducing mechanical cues into intracellular signaling cascades that control cell migration, differentiation, and survival. Mammalian cells possess at least 24 integrin heterodimers. In most cases, the molecular mechanisms by which different integrins fulfill their distinct physiological roles remain poorly understood. In particular, relatively little is known about how different integrin heterodimers may function in the context of mechanotransduction. Here, we use single-molecule fluorescent molecular tension sensors (MTSs) to measure the forces experienced by $\alpha_5\beta_1$ and α_v -class integrins, two classes of fibronectin-binding integrins that are thought to play distinct roles in cell adhesion and mechanotransduction. We developed MTSs that incorporate the full 9th and 10th type III fibronectin domains, here termed MTS_{FN9-10}. Importantly, these probes include a secondary binding site, termed the synergy site, which is specifically required for maximal binding strength and activation for $\alpha_5\beta_1$ but not α_v -containing integrins. We observe that fibroblasts seeded on MTS_{FN9-10} form large, plaque-like adhesions rich in $\alpha_5\beta_1$ integrins, a phenotype that is lost in the absence of a functional synergy site. At the single-molecule level, preliminary data indicate that the force per integrin lies between 1 to 5 pN for cells adhering to MTS_{FN9-10}-coated surfaces, a range similar to that observed for cells adhering to MTSs containing only a linear RGD binding epitope. In ongoing work, we use MTS_{FN9-10} and related probes to parse out the contributions of the synergy site to traction force generation and force sensing at both $\alpha_5\beta_1$ and α_v -containing integrin complexes.

P1431**Board Number: B880****Rigidity sensing by tropomyosin-regulated nanometer steps in local contractions.**H. Wolfenson¹, G. Meacci¹, S. Liu¹, M.R. Stachowiak², T. Iskratsch¹, S. Ghassemi¹, P. Roca-Cusachs^{3,4}, B. O'Shaughnessy², J. Hone¹, M.P. Sheetz^{5,6};¹Columbia University, New York, NY, ²Department of Chemical Engineering, Columbia University, New York, NY, ³Institute for Bioengineering of Catalonia (IBEC), Barcelona, Spain, ⁴University of Barcelona, Barcelona, Spain, ⁵Mechanobiology Institute, National University of Singapore, Singapore, NY, ⁶Biological Sciences, Columbia University, New York, NY

The rigidity of the extracellular matrix (ECM) affects processes such as cell migration, apoptosis, proliferation, and differentiation. To sense the rigidity of the ECM, the leading edge of the cell protrudes forward, encounters a new matrix, and pulls on it via nascent integrin adhesions. At its most basic sense, rigidity sensing is taken as the decision to reinforce the adhesions during the initial period of force

application. Thus, with increased rigidity, cells build stronger adhesions that can withstand higher forces. This indicates the existence of a tight regulation mechanism between rigidity, force production, and adhesion reinforcement, all occurring at the sub-micrometer level within a short time window of up to few tens of seconds. However, it is unknown how this rigidity/force feedback loop is regulated. Addressing this question requires ultra-high resolution analysis of cellular forces, both in time and space. Thus, in the current work, we measured myosin force production within cells at the nanometer and millisecond level during rigidity sensing. Using arrays of Polydimethylsiloxane (PDMS) micropillars as substrates for spreading of mouse embryonic fibroblasts, we found that local contractile units that bear resemblance to sarcomeres pulled opposing pillars in nano-level myosin-II-generated step-wise contractions. Surprisingly, cells produced the same step size of 2.1-2.4 nm, regardless of rigidity. What correlated with rigidity was the number of steps taken before reaching a ~20 pN force level, which activated the recruitment of α -actinin to the pillars. The constant step size suggested a structural restriction for the myosin motors; indeed, when we knocked-down tropomyosin-1 (a known a regulatory protein that wraps around actin filaments), the step-wise movements were dramatically altered and larger steps were observed. Further, after tropomyosin-1 knock-down, the cells could not sense the rigidity properly, indicating that tropomyosin-1 is directly involved in rigidity sensing. Importantly, tropomyosin-1 was previously implicated as a tumor suppressor whose extremely low expression levels in malignant cells allows them to grow on soft agar (i.e., anchorage-independent growth). When we tested a non-malignant vs. malignant cell line, we verified that the absence of tropomyosin correlated with higher forces, suggesting that anchorage-independent growth depends upon the production of high forces that activate the signaling cascades required for proliferation even on soft matrices.

P1432

Board Number: B881

Integrin-beta3 clusters recruit clathrin-mediated endocytic machinery in the absence of traction force.

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Matrix-integrin activation triggers cell adhesion. Force development at integrins can differentially regulate adaptor protein recruitment, and micro-partitioned RGD-membrane provides a unique substrate to study force-dependent signal transduction. Previously, we have used RGD-membrane to investigate initial activation of integrin clustering and podosome formation. Here we report that clathrin-associated adaptor protein Dab2 progressively binds to integrin-beta3 after 20-min of cell adhesion on mobile RGD-membrane. When a single cell adheres on the substrate with both mobile and immobile ligands, Dab2 is preferentially recruited to integrin-beta3 at mobile RGD regions. Intriguingly, Dab2 is mutually excluded with other integrin-binding proteins, including talin, kindlin, focal adhesion

kinase, paxillin, and vinculin. Point mutations of tyrosine residues of integrin-beta3 block Dab2 binding. Increase cellular contractility by RhoA-Q63L and phospho-mimic MRLC mutant impedes Dab2 recruitment on mobile RGD membrane. More importantly, decrease myosin-II activity by blebbistatin promotes Dab2 binding to integrin-beta3 on immobile matrices. As a result, Dab2 recruits clathrin-mediated endocytic machinery and results in RGD-integrin endocytosis. We propose that absence of traction force causes recruitment of Dab2/clathrin and endocytosis of integrin-beta3.

P1433

Board Number: B882

Low adhesive scaffold collagen, inducing spheroid formation, promotes the cell crawling of fibroblasts.

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[Background] Fibroblasts exist in the connective tissues and are generally separated by extracellular matrices (ECMs). One of functions in fibroblast is synthesis, secretion, and deposition of the ECM proteins such as collagen molecules. In addition, fibroblasts can migrate in connective tissues for remodeling and renewal. In vitro, we observe that cell crawling, associated with the actin polymerization, is a highly complex integrated process. However, the molecular mechanism of crawling still remains to be clarified. We succeeded in developing low adhesive scaffold type I collagen (LASCOL) by enzyme treatment (patent pending), and we found that fibroblasts cultured on LASCOL spontaneously form an aggregated sphere body (spheroid). In this study, we report the cell motility and the expression patterns of mRNAs of fibroblast spheroid. [Materials & Methods] We purchased pepsin-treated collagen (Pep-Col) and obtained LASCOL by enzyme treatment of pig type I collagen. The culture dish (φ35 mm) was coated with LASCOL solution or Pep-Col solution. Subsequently, mouse NIH/3T3 cells or normal human dermal fibroblasts (NHDF) were cultured on each dish. After 24 hrs, to confirm spheroid formation, we observed cell morphology by using a phase-contrast microscope. To investigate the cell motility, we stained filamentous actin (F-actin) with DyLight 555-labeled phalloidin. To further clarify the relationship between spheroid formation and epithelial mesenchymal transition (EMT), we stained spheroid with DyLight 555-labeled polyclonal antibodies specific against EMT molecular markers. Finally, cell crawling was monitored for a period of 24 hrs and analyzed by a time-lapse observation with a VW-9000PC software (KEYENCE, Japan). [Results] Each of NIH/3T3 and NHDF cells formed the spheroid morphology by culturing on the LASCOL scaffold. By SEM observation, single cells cultured on Pep-Col adhered strongly on the scaffold, showing flat and smooth appearance. In contrast, we showed that a lot of protrusions existed on the surface of NIH/3T3 cells of spheroid. F-actin was highly expressed in each cell of spheroid, as compared with a single flat cell. Interestingly, the crawling rate was drastically accelerated by formation of spheroid. In conclusion, we demonstrated that the adherence of fibroblasts to the LASCOL scaffold is weak, and cells gather and adhere each other to form spheroid. [Funding] This

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P1434

Board Number: B883

ECM Stiffness Enhances Integrin Signaling to Alter Tumor Cell Metabolism.

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Tumors exhibit altered cellular metabolism supportive of continuous cell growth. Nevertheless, the fundamental molecular mechanisms through which altered metabolism regulate tumor behavior remain unclear. We previously demonstrated that elevated ECM stiffness and epithelial cell tension drive squamous carcinoma and mammary transformation, identifying integrin signaling as a key conduit of ECM mechanical cues and a potent regulator of pro-tumorigenic signaling. We hypothesized that elevated ECM stiffness potentiates cell metabolism by increasing the expression and activity of key metabolic targets driven by enhanced pro-tumorigenic signaling via integrin activity to regulate malignant progression. To test this hypothesis, we profiled metabolic markers using a combination of H1 NMR, quantitative RT-PCR, immunoblotting and immunofluorescence of MMTV-PyMT transgenic tumors (\pm ECM crosslinking) and breast tumor mammary epithelial cells grown on tunable hydrogel substrates in vitro. To determine the role of integrin signaling, we inhibited integrin signaling used small molecule inhibitors and, conversely, facilitated integrin signaling through ectopic expression of a β 1 integrin clustering construct. We used pharmacological inhibitors targeting glucose uptake, glycolysis, lactate transport, or oxidative phosphorylation to assess the interplay between ECM stiffness, metabolism and tumor cell behavior, including invasion, migration, and proliferation. Our data suggests that ECM stiffening induced elevated tumor cell glycolysis downstream of integrin signaling by targeting key regulators of glycolysis such as the glucose and lactate transporters and lactate dehydrogenase. Additionally, inhibitors of metabolism abrogated tumor cell proliferation and invasion in vitro and in vivo. These data suggest that elevated ECM stiffness drives tumor progression through modulation of tumor cell metabolism. Additional studies are underway to further clarify how ECM-driven metabolic dysregulation drives tumor behavior.

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P1435**Board Number: B884****Integrin Signaling via Traction Forces Regulates Early Endoderm Specification.**H. Taylor-Weiner¹, N. Ravi¹, A.J. Engler¹;¹Bioengineering, University of California, San Diego, La Jolla, CA

Morphogenic events during early development, including gastrulation, require force-mediated motility and coincide with initial fate specification of embryonic stem cells (ESCs), resulting in the formation of the definitive endoderm (DE) and mesoderm layers of the trilaminar embryo. Pluripotency also inhibits contractility, suggesting a switch that is initiated upon differentiation. However, the onset of contractility during differentiation and whether integrin-signaling regulates fate choices is not well understood. To address the requirement of traction forces for differentiation, we examined mouse ESCs grown in DE inductive medium on fibrillar fibronectin labeled with a FRET probe to assess deformation. We found that inhibition of contractility via blebbistatin, an inhibitor of non-muscle myosin, prevented expression of the DE marker SOX17. When blebbistatin was removed, DE induction medium was sufficient to activate ESC traction forces, resulting in a decrease in the observed fibronectin FRET intensity ratio. By contrast ESCs grown in pluripotency medium did not exert significant tractions against the FN matrix. Laminin isoforms have been reported to improve DE induction efficiency, but it is not clear whether traction forces mediate this effect. Mouse ESCs grown in DE inductive medium on fibrillar matrices with varying amounts of laminin-111 decreased their fibronectin traction forces in a laminin-dependent manner, suggesting preferential binding to laminin over fibronectin. When the laminin-binding alpha3 integrin was blocked, TGF-beta signaling was inhibited by increased Smad7 expression, and the laminin effects on traction forces and differentiation were reversed. These data imply that traction forces and integrin-signaling are important regulators of early fate decisions in ESCs.

P1436**Board Number: B885****DNA nanostructures as a nanometer-scaled patterned substrate for controlling cell adhesion.**J. Fern¹, D. Kim², D. Wirtz^{2,3}, R. Schulman^{1,4};

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Integrins and other cell surface receptors involved in cell adhesion play a crucial role in cancer cell migration, survival and tumor resistance to anti-cancer pharmaceuticals [1]. Knowledge of how these receptors sense multiple signals in the extracellular environment to influence cell adhesion and migration is important to understanding tissue organization as well as how failures in these organizational processes underlie disease [1]. One of the most salient features of the sensing process is

that cells are sensitive not only to types of chemical cues but also to, among other properties, the density of chemical ligands and their spacing at the nano- and microscale [2]. These interactions have been elucidated by studies in which these variables are tightly controlled and easy to observe within the cell's environment. Currently, most studies investigating how cells integrate integrin signals use only a single type of adhesion peptide (e.g. RGD) or full extracellular matrix proteins such as collagen or fibronectin [2]. In the latter case, multiple ligands are presented to cells, but their density and spacing are difficult to control. To understand quantitatively how cells integrate information about multiple ligands, there is a need for an assay enabling control over the nanoscale spacing and density of different types of cell adhesion ligands within a single environment.

We have developed a method to control the density and nanoscale spacing of multiple types of ligands using two-dimensional DNA origami nanostructures. The DNA origami method involves folding a long single strand of DNA into a specified structure using multiple short DNA strands into a 2 nm-high template structure bearing about 200-250 DNA sequence addressable binding sites for peptides and other small molecules such that the binding sites' positions are known to a 3-6 nm resolution [3]. The DNA origami structures we have can position peptide ligands on a surface with controlled density and nanoscale spacing. Fluorophores on the DNA origami enable these ligands to be localized precisely and biotins attached to the templates anchor the structures securely to PEGylated coverslips using Neutraavidin-biotin binding chemistry.

As a proof of concept, we show that mouse embryonic fibroblast (MEF) cells bind specifically to DNA origami nanostructure substrates that present RGD ligands and not to DNA origami nanostructures with no attached ligands or to PEG surfaces alone. For origami nanostructures presenting RGD ligands, the fraction of cells that spread as well as their average area is controlled by DNA nanostructure/RGD surface density. Future studies will investigate how nanostructures with a heterogeneous nanopattern of cell adhesion ligands influence the cell adhesion process.

[1] Desgrosellier J.S., Cheresh D.A., Nat. Rev. Cancer 10, 9–22 (2010).

[2] Geiger, B., Spatz, J.P., Bershadsky, A.D., Nat. Rev. Mol. Cell Biol. 10, 21-33 (2009).

[3] Rothmund, P. W. K., Nature 440, 297–302 (2006).

P1437

Board Number: B886

Stiff substrates enhance leukocyte recruitment from fluid flow.

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Many inflammatory diseases such as atherosclerosis, obesity, and diabetes, are driven by the excessive recruitment of circulating monocytes, which differentiate into macrophages and can promote chronic

inflammation. Monocytes exit the bloodstream through a multistep process called the leukocyte adhesion cascade, in which they first attach to endothelial cells by binding selectins, then roll across the endothelium until integrin-based adhesions promote cell arrest, and finally they transmigrate through the vessel wall. This process has been widely studied both in vitro and in vivo and is known to depend on both shear stress and protein density. However, very little is known about whether the stiffness of vessel walls could also play a role in leukocyte adhesion, which is surprising given that arterial stiffening is a known predictor of cardiovascular mortality and increases with age, smoking, obesity, and diabetes. In addition, stiff substrates have been shown to strengthen integrin-based adhesions in cells under static conditions (without fluid flow), and both selectins and integrins are known to exhibit force-dependent, catch bond behavior. In this study, we investigated whether substrate stiffness could directly alter the adhesion of monocytes from flow by using an in vitro model for blood flow, in which human monocytic THP1 cells were perfused across polyacrylamide gels with elastic moduli ranging from that of healthy arteries (1-5 kPa) to diseased arteries (24-84 kPa). To enable cell attachment, the gels were functionalized with prescribed densities of recombinant P-selectin or E-selectin, which both bind the ligand PSGL-1 on the surface of leukocytes. Interestingly, we found that cell attachment from fluid flow (at 100s^{-1}) did not depend on substrate stiffness when the gels were coated with P-selectin alone. On the other hand, coating gels with E-selectin revealed a strong dependence on substrate stiffness, in which more cells attached to stiff gels than soft gels. In addition, stiff gels supported firm cell arrest at high E-selectin densities, while soft gels promoted continuous cell rolling. These contrasting results of stiffness-independent adhesion through P-selectin binding and stiffness-dependent adhesion through E-selectin binding may be due to the fact that E-selectin engages other ligands besides PSGL1, including CD44 and ESL-1. Another possibility is that structural differences between P-selectin and E-selectin lead to different force-dependent bond behavior. Overall, our results demonstrate that E-selectin-mediated capture of THP1 cells from flow is enhanced by substrate stiffness, while P-selectin-mediated capture is stiffness-independent. The goal of future work is to investigate whether this also holds true for primary leukocytes.

P1438

Board Number: B887

Cell Biological Analysis of VRK1 and its Role in Regulating Cell Proliferation and Migration.

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Vaccinia-related kinase (VRK1) is a nuclear ser/thr kinase that is considered to be “pro-proliferative”; it is highly expressed in proliferative tissues and several cancers. Moreover, VRK1 expression turns off as cells enter G0 and as mammary epithelial cells form differentiated, non-proliferative acini in 3D culture. We have shown that VRK1 depletion significantly slows proliferation of both normal and malignant mammary epithelial cells in 2D culture. Furthermore, VRK1 has been identified by others as one of the

22 genes whose expression is down-regulated during normal acinus formation in a 3D culture system. Our lab has also shown that depletion of VRK1 in malignant MDA-MB-231 cells reduces tumor size and the incidence of distal metastases in engrafted mice.

VRK1 has been identified as part of a 19 gene signature whose overexpression is correlated with poor prognosis in a subset of breast cancers. We have taken several approaches to the analysis of how constitutive and/or elevated VRK1 expression might alter cell behavior. We have studied control MCF10a cells and a matched cell line that constitutively expresses elevated levels of 3xFlag-VRK1 from a CMV promoter. In conventional 2D culture, VRK1 overexpression did not affect the proliferation rate of cells cultured in either 5% or 0.25% serum. However, in 3D culture, we found that VRK1-overexpressing MCF-10a cells form enlarged and distorted acini compared to control cells. These data suggest that VRK1 overexpression may modulate cellular survival, proliferation, polarity and/or migration in the presence of an extracellular matrix (ECM). Our recent microarray analysis of control vs. VRK1-overexpressing cells revealed that several proteins involved in cell:cell and cell:matrix interactions, as well as proteins involved in cell migration, are upregulated in VRK1-overexpressing cells. We have examined the potential roles of VRK1 in adhesion and spreading in the absence or presence of ECM proteins (collagen, fibronectin, laminin). We observed that VRK1 overexpression slows the rate of cell spreading, although it has no impact on cell adhesion. Finally, we assessed cell migration in vitro using a wound healing assay; surprisingly, we found that overexpression of 3xFlag-VRK1 significantly decreased the rate of sheet migration. In sum, we conclude that VRK1 overexpression can exert an influence on cell proliferation, spreading and migration, which may have its contribution to tumorigenesis.

P1439

Board Number: B888

Integrins and dystroglycan compensate each other to mediate BM assembly and epiblast morphogenesis.

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Cell-extracellular matrix (ECM) interactions in general, and cell-basement membrane (BM) interactions in particular, regulate cell survival, proliferation, differentiation and polarity, which are all essential for tissue development and maintenance. Integrin and dystroglycan (DG) are major cell surface receptors that link the BM to the actin cytoskeleton. Integrins also activate intracellular signaling pathways. While it has been previously thought that DG primarily serves as a mechanical link, there are data that argue for a signaling role as well. However, identification of unique signaling roles arising from DG has been hampered because of compensation from integrin contributions. This question has been particularly difficult to study in tissues because of the presence of several classes of interacting integrins. In our studies of BM-receptor interactions during epithelial morphogenesis, we employ embryonic stem (ES) cell-derived embryoid bodies (EBs). EB tissue is structurally similar to the peri-implantation embryo

tissue. We found that ablation of integrin beta1 blocks endoderm formation and BM assembly. Treatment of integrin beta1-null EBs with 100 microg/ml laminin-111 induces the assembly of a BM on the EB surface and promotes epiblast differentiation and polarization without recruitment of “focal adhesion” proteins to the cell-BM interface. The data strongly indicate that DG is capable of mediating epithelial differentiation in the complete absence of integrins. To test this hypothesis, we generated integrin beta1/DG double knockout ES cells. Analysis of EB differentiation demonstrated that ablation of both integrin beta1 and DG completely eliminates laminin-induced BM assembly and epiblast epithelial differentiation. Blocking experiments using alpha-DG neutralizing antibody that recognizes the ligand-binding carbohydrate epitope suggest that the BM-DG interaction is required for epiblast polarization. In addition, incubation of integrin beta1-null EBs with laminin and F3, a laminin alpha1-agrin hybrid protein that enhances laminin-DG interactions, significantly increases BM assembly. Altogether, these results suggest that integrins and DG compensate each other to mediate BM assembly and epiblast differentiation. The ligand binding activity of DG is required for its functions in the absence of integrins.

P1440

Board Number: B889

Cell traction force and mechanosensing in cell–substrate interaction.

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Cells constantly probe their surrounding microenvironment by pushing and pulling on the extracellular matrix (ECM). While it is widely accepted that cell induced traction forces at the cell-matrix interface play essential roles in cell signaling, cell migration and tissue morphogenesis, a number of puzzling questions remain with respect to mechanosensing in cell-substrate interactions. Here we show that these open questions can be addressed by modeling the cell-substrate system as a pre-strained elastic disk attached to an elastic substrate via molecular bonds at the interface. Based on this model, we establish analytical and numerical solutions for the displacement and stress fields in both cell and substrate, as well as traction forces at the cell-substrate interface. We show that the cell traction generally increases with distance away from the cell center and that the traction-distance relationship changes from linear on soft substrates to exponential on stiff substrates. These results indicate that cell adhesion and migration behaviors can be regulated by cell shape and substrate stiffness. Our analysis also reveals that the cell traction increases linearly with substrate stiffness on soft substrates but then levels off to a constant value on stiff substrates. This biphasic behavior in the dependence of cell traction on substrate stiffness immediately sheds light on an existing debate on whether cells sense mechanical force or deformation when interacting with their surroundings. Finally, it is shown that the cell induced deformation field decays exponentially with distance away from the cell. The characteristic length of this decay is comparable to the cell size and provides a quantitative measure of how far cells feel into the ECM.

References

1. He, S., Y. Su, B. Ji, and H. Gao. 2014. Some basic questions on mechanosensing in cell–substrate interaction. *Journal of the Mechanics and Physics of Solids* 70:116-135.
2. Zhong, Y., and B. Ji. 2013. Impact of cell shape on cell migration behavior on elastic substrate. *Biofabrication* 5:015011.
3. Zhong, Y., and B. Ji. 2014. How do cells produce and regulate the driving force in the process of migration? *Eur. Phys. J. Spec. Top.* 223:1373-1390.

P1441**Board Number: B890****Axo-glia interaction preceding CNS myelination is regulated by bidirectional Eph-ephrin signaling.**L.S. Laursen¹, C. Linneberg¹;¹Department of Molecularbiology and Genetics, Aarhus University, Aarhus, Denmark

In the central nervous system, myelination of axons is required to ensure fast saltatory conduction and for survival of neurons. However, not all axons are myelinated, and the molecular mechanisms involved in guiding the oligodendrocyte processes towards the axons to be myelinated are not well understood. Only a few negative or positive guidance clues that are involved in regulating axo-glia interaction prior to myelination have been identified. One example is laminin, known to be required for early axo-glia interaction, which functions through $\alpha6\beta1$ integrin. Here we identify the Eph-ephrin family of guidance receptors as novel regulators of the initial axo-glia interaction, preceding myelination. We demonstrate that so-called forward and reverse signaling, mediated by members of both Eph and ephrin subfamilies, has distinct and opposing effects on processes extension and myelin sheet formation. EphA forward signaling inhibits oligodendrocyte process extension and myelin sheet formation, and blocking of bidirectional signaling through this receptor enhances myelination. Similarly, EphB forward signaling also reduces myelin membrane formation, but in contrast to EphA forward signaling, this occurs in an integrin-dependent manner, which can be reversed by overexpression of a constitutive active $\beta1$ -integrin. Furthermore, ephrin-B reverse signaling induced by EphA4 or EphB1 enhances myelin sheet formation. Combined, this suggests that the Eph-ephrin receptors are important mediators of bidirectional signaling between axons and oligodendrocytes. It further implies that balancing Eph-ephrin forward and reverse signaling is important in the selection process of axons to be myelinated.

P1442**Board Number: B891****Rupture behaviors of receptor-ligand bond at ultralow loading rate.**D. Li¹, B. Ji¹;¹Biomechanics and Biomaterials Laboratory, Department of Applied Mechanics, Beijing Institute of Technology, Beijing, China

The rupture behaviors of molecular bonds at the ultralow loading rates have recently intrigued intensive attentions because of its scientific importance as well as its distinct features compared with those at the faster loading rates. It was observed that many so-called “mechanical” proteins exhibit a nonzero asymptotic strength limit of unfolding at a vanishing loading rate. And the mean rupture force of single receptor-ligand bond shows a similar asymptotic manner. This study is to understand the rupture behaviors of molecular bond at ultralow loading rate by using both theoretical analyses and Brownian dynamic simulations. We showed that when the loading rate is smaller than a critical value the rupture force becomes no longer dependent on the loading rate, implying a nonzero strength of molecular bond at a quasi-static loading. We found that this saturation behavior is caused by bond rebinding at the ultralow loading rate. We further derived an analytical solution of the limiting loading rate below which the bond rebinding dominates the rupture process. This solution will assist us to determine which DFS data are valid for calculating the properties of energy landscape by fitting the $F \sim \ln(Kv)$ plot using conventional methods, and thus to improve the predictions. It would also provide guidelines for preparing DFS experiments and simulations on how to choose the loading rates according to the stiffness of loading devices.

References

1. Li, D., and B. Ji. 2014. Predicted Rupture Force of a Single Molecular Bond Becomes Rate Independent at Ultralow Loading Rates. *Physical Review Letters* 112:078302.

Cell–Cell Junctions 2**P1443****Board Number: B892****Unraveling the Life and Times of Gap Junctions: Comparison of Gap Junction Structures with High Resolution, Transmission and Light Microscopy.**S.A. Murray¹, M.M. Steed², T.I. Shakespeare³, B. Nickel¹, M.E. Bisher¹;¹Cell Biology, Univ Pittsburgh Sch Med, Pittsburgh, PA, ²Biology, Winston-Salem State University, Winston-Salem, NC, ³Biology, Fort Valley State Univ, Fort Valley, GA

In most of the cells of the body, cellular function is thought to be modulated by cell-cell communication through gap junction channels. This communication is regulated by the rate of delivery of gap junction channel proteins to the plasma membrane, gap junction channel assembly, aggregation of these

channels into gap junction plaques, the subsequent opening or closing of these channels and the removal of these channels from the cell surface. It is clear that channels can be removed by internalization of the entire gap junction plaques or fragments of the plaques by an endocytosis process that results in the formation of spherical annular gap junction vesicles. To unravel the molecular machinery needed for formation, endocytosis and the processing of annular gap junctions, reliable and efficient methods for visualizing and distinguishing gap junction structures are critical. In this study, we use conventional and high resolution light microscopy, transmission electron microscopy, and time lapse imaging to positively identify gap junction structures and to characterize the details of their formation, internalization to form annular gap junctions, and the intricate processing needed for degradation. In the past, gap junction puncta seen with conventional immunocytochemical light microscopy have been classified as annular gap junctions mainly based on the size of the puncta. Here we demonstrate that size alone is not enough to distinguish annular gap junction vesicles from secretory vesicles and establish that some of the vesicles that would have been classified as secretory vesicles are really annular gap junctions. Annular gap junctions were shown to undergo a unique fission process that results in the release of small buds from the vesicle as well as in some cases the division of the annular gap junction vesicle into two smaller vesicles. Information on the processing of annular gap junction vesicles is critical to the understanding of gap junction turnover and degradation. Methods to analyze the details of annular gap junction processing will add in our understanding of gap junction function. Grants: NSF #MCB-1408986 and #NIH 5T36GM008622

P1444

Board Number: B893

The structure and activity of the gap junction Nexus signaling complex is regulated by the C-terminus of connexins.

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Gap junction connections perform both essential channel and non-channel functions. These functions are regulated by posttranslational modifications that are primarily applied to the carboxyl-termini (CT) of connexins that comprise the dodecameric gap junction channels. The different connexins vary widely in the amino acid sequences and length of their cytoplasm-localized CTs. Connexin CTs bind numerous other proteins, where binding depends on the CT sequence and on posttranslational modifications. Gap junctions and their binding partners, therefore, make up a specialized signaling structure that we termed the Nexus.

We report new aspects of the gap junction Nexus that we uncovered using fluorescence recovery after photobleach (FRAP) and other imaging techniques. Specifically, we found that the amino acid residues within the CT of connexin 43 (Cx43) beyond amino acid 257 are necessary and sufficient to establish an extremely stable arrangement of gap junction channels within the plaque. Analysis of mobility of connexin sectional deletions, single amino acid mutations, and hybrid connexin-type mutant channels

with live confocal microscopy revealed that a cysteine residue at position 260 (of 381 total amino acids) is sufficient to stabilize the Nexus.

Cysteine residues in the CT of other receptors and channels regulate the formation of membrane-associated structures (e.g. aquaporins and glutamate receptor subunits). The extreme CT of Cx32 has been previously reported to be palmitoylated. Therefore, we used FRAP to compare the mobility of other connexins with potentially palmitoylated cysteines (Cx32 and Cx47) in the CT and those without cysteine residues in the CT (Cx26 and 30) fused to new-generation fluorescent proteins in conjunction with the FRAP technique. We will continue to test the hypothesis that palmitoylation controls Nexus stability using pharmacological inhibitors of palmitoylation.

These findings are very clearly demonstrated through both qualitative and quantitative analysis of FRAP experiments and other optical techniques. This includes quantitative analysis of four dimensional microscopy with FRAP experimentation and video representations of time-lapse 3D reconstruction of photoconvertible proteins within the Nexus.

This work was supported by the following grants: 5T32NS007439-14, NS041282.

P1445

Board Number: B894

Gold Nanoparticle-Mediated Laser Perforation as a New Method to Analyze Dye Transfer and Gap Junctional Coupling.

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We present the possibility to use the gold nanoparticle-mediated (GNOME) laser perforation (LP) method for a quantitative high throughput analysis of gap junction coupling in a cell monolayer as an advantageous alternative to the scrape loading/dye transfer (SL/DT) technique. During SL/DT membrane impermeable but gap junction permeable dyes like Lucifer Yellow (LY) are introduced into a cell monolayer by the mechanical opening of cells with a sharp razor. During GNOME LP, the interaction of a laser beam with large, membrane-adhered gold nanoparticles (200 nm) results in a heat-generated effect which leads to the permeabilization of cell membranes, thereby allowing the introduction of the dye into the cells. In our experiments, we applied the laser beam with 88 μm in diameter on GM-7373 endothelial cells grown to a monolayer on a tissue culture dish. After LY incubation we observed a LY positive cell band of 179 μm . The width of this LY positive cell band was reduced to 95 μm when the cells were treated with carbenoxolone (CBX) indicating that a) inhibition of diffusion through gap junctions only leads to LY-loaded cells encompassed by the laser beam and b) the large band of LY positive cells observed in absence of CBX was due to dye diffusion through gap junctions. Moreover, a forskolin-induced enhancement of gap junction coupling previously shown by the mechanical SL/DT

could be observed using the GNOME LP. Taken together, the results show that the GNOME LP can be used to study the cellular gap junction coupling, similar to SL/DT. The GNOME LP offers the advantage that it can be performed by a computerized system when cells cultivated in a multiwell plate are mounted onto an automatized microscope stage. Additionally, the fluorescence imaging can be performed automatically and is followed by a semi-automatic quantification of the images using a java-based ImageJ plugin. The automation of the process represents a gain of time and accuracy in experimentation that could not be achieved for mechanic SL/DT experiments because of the variability of the mechanical scraping. Furthermore, the usage of SL/DT technique is limited only to cells that strongly adhere to the culture surface. The permeabilization of the cells by GNOME LP is a non-contact process and could be applied on cells such as the rat brain endothelial cells RBE-4, which form a monolayer that did not support the mechanical SL/DT. Taken together the report shows that the GNOME LP is a promising technique for a high throughput analysis of gap junction coupling in cell monolayers.

P1446

Board Number: B895

Fusion to fluorescent proteins causes previously unrecognized effects on gap junction mobility and Nexus arrangement.

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Gap junctions are comprised of connexins that form channels connecting adjacent cells. They also act as the core of a signaling complex called the Nexus. Much insight into the structure and dynamic morphology of the Nexus has been gained from the use of electron and optical microscopy. Fluorescent protein fusions to connexins are important tools for studying the unusually rapid, responsive, and regulated cycle of connexin production, channel formation, channel regulation and degradation.

We used connexin-fluorescent protein fusions with live confocal microscopy to perform fluorescence recovery after photobleach (FRAP) experiments. We used new-generation fluorescent proteins to reveal two previously unrecognized qualitative characteristics of the Nexus: A) Plaque formation does not require plaque stability; B) Mixing of different connexin types within the Nexus is affected by the type and site-of-attachment of fluorescent proteins. Specifically, we find that the degree of mixing of different gap junction types varies not only with connexin type as previously described, but also varies according to the fusion-site of the fluorescent protein tag. We found that two immobile connexins can remain separated into distinct parts of the same gap junction plaque, as previously described. However, when we co-transfected stable connexin 43 (Cx43) with more mobile connexins (superfolderGFP-Cx30 or Cx26-mVenus, and others) the gap junctions mixed when included in the same gap junction Nexus structure.

We found that Cx26 and Cx30 gap junctions are significantly more mobile than Cx43. We also found that Cx26 is significantly more mobile when tagged by the monomerized superfolderGFP (msfGFP) variant

(V207K) than the non-monmerized superfolder (GFP 43.5+/-10% and 6+/-2% normalized FRAP at 30 seconds post-bleach for Cx26-msfGFP and Cx26-sfGFP, respectively). This effect was attenuated but still significant when the fluorescent protein tag is fused to the N-terminus of Cx26 instead of the C-terminus. The effects of the fluorescent protein tag were similar for Cx30. However, Cx43 displays very little or no mobility regardless of fluorescent protein type or site of fusion to a fluorescent protein.

We used several methods to quantitatively compare the mobility of gap junctions using data generated in FRAP experiments. We find that the unique morphology of the Nexus requires that results obtained through standard single focal plane FRAP experimentation be verified by 3D-timelapse imaging. These results have major implications for our understanding of the gap junction Nexus structure. Supported by the following grants 5T32NS007439-14, NS041282.

P1447

Board Number: B896

K63-linked poly-ubiquitination and phosphorylation: signals that regulate connexin43 gap junction turnover.

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Intercellular communication is a vital function for cellular homeostasis. Gap junctions (GJs) serve as a mechanism for cell-cell communication, physically coupling cells together and allowing small molecules to pass from cell to cell. Connexin (Cx) proteins are the transmembrane components of GJs. It is known that connexin 43 (Cx43) GJs are regulated via ubiquitination and phosphorylation. Ubiquitination is a post translational modification in which one or more 8.5 kDa ubiquitin (Ub) moieties are covalently attached to lysines on target proteins and is characterized by us and others on GJs and internalized GJ vesicles (annular GJs) (Rutz & Hulser 2001; Leithe & Rivedal 2004; Girão et al. 2009, Ribeiro-Rodrigues et al. 2014). Different types of ubiquitination can target proteins and protein complexes to cellular degradation pathways (e.g. K48-linked, proteasomal; K63-linked, clathrin-mediated endocytosis, endo-/lysosomal, autophagosomal). Phosphorylation of Cx43 has been shown to be a regulatory mechanism for Cx43 trafficking, GJ assembly, gating and GJ internalization and degradation (Lampe & Lau, 2004; Warn-Cramer & Lau, 2004; King & Lampe, 2005; Laird, 2005; Moreno, 2005; Solan & Lampe, 2005; 2007; 2009). These phosphorylation events lead to conformational changes within the C-terminus of Cx43 that may allow for regulatory protein binding. Little is known about what types of Ub modify Cx43 and how ubiquitination and phosphorylation coordinate to regulate GJ internalization and degradation. Using immunological and biochemical analyses we show that Cx43 is K63-polyubiquitinated in both GJs and annular GJs in cells expressing Cx43 endogenously (PAEC) and exogenously (HeLa, MDCK). Mutating specific lysines within the C-terminus of Cx43 leads to significant increases in total plasma membrane GJ number, half-life and phosphorylation, indicating GJ internalization impairment. Cx43 K to R mutants exhibit increases in several critical serine phosphorylations, indicating a prerequisite of both phosphorylation and K63-polyUb for internalization. We hypothesize that K63-linked polyubiquitination of Cx43 following phosphorylation is necessary for internalization and autophagic degradation of GJs.

P1448**Board Number: B897****Expression and Regulation of Connexins under “Inflammatory” state: Communication between Human Intestinal Epithelial Cells and Immune Cells.**S. Ghadban¹, M.E. El-Sabban²;¹Anatomy, Cell Biology and Physiological Sciences, American Univ Beirut, Beirut, Lebanon, ²American Univ Beirut, Beirut, Lebanon

Inflammatory bowel diseases (IBD) are related to functional impairment of intestinal epithelial cells (IECs) due to infiltration of the sub-mucosa by inflammatory cells. Three essential mechanisms may contribute to the induction of this state: (i) soluble mediators secreted by inflammatory cells, (ii) direct adhesion and signaling molecules expressed on the surface of immune cells and epithelial cells and (iii) cytoplasmic exchange of specific signals between the inflammatory cells and IECs via gap junction (GJ) channels. We explored the nature of the interaction between human IECs and macrophages (MΦ) in an in vitro model of IBD. We investigated the role of inflammatory cells in inducing IECs Matrix Metalloproteinases (MMPs) as a mechanism that results in breaching the sub-epithelial basement membrane and bringing inflammatory cells in close proximity with IECs. We then identified potential adhesion molecules and connexins (Cxs) involved in intercellular communication and studied the effect of inflammatory mediators on connexins expression at the transcriptional, protein, cellular localization, and functional levels. IECs and MΦ express Cx26, Cx43, and Cx45; however, Cx32 was only expressed in IECs. Connexin 26 and Cx43 expression and MMPs enzymatic activity were significantly up regulated in IECs under inflammatory conditions, which re-sulted in enhanced functional IECs-MΦ intercellular communication. We propose that the combination of paracrine and hetero-cellular communication between IECs and MΦs play a pivotal role in the regulation of epithelial cell function by establishing junctional complexes between inflammatory cells and IECs that might stabilize leakiness of intestinal epithelial barrier function.

P1449**Board Number: B898****Signals and Mechanisms Regulating Endocytosis Across the Connexin Protein Family.**C.G. Fisher¹, M.M. Falk¹;¹Biological Sciences, Lehigh University, Bethlehem, PA

Gap junctions are clusters of channels that directly connect the cytoplasm of apposing cells and permit the passage of hydrophilic molecules less than approximately 1.5kD in size. The channels are comprised of proteins called connexins (Cxs) which are four-pass transmembrane spanning proteins with their N- and C-termini residing in the cytoplasm. Twenty-one connexins exist in the human genome and are

expressed tissue specifically. In terms of controlling cell-cell communication, recent research has shown that the regulated endocytosis of gap junctions is as important for their function as the ability of cells to open and close existing channels; and that mis-regulated gap junction endocytosis can cause disease. Thus it is crucial to understand how and under what conditions cells use endocytosis to remove gap junctions from the plasma membrane. Previous work has shown that Connexin43 (Cx43), the most commonly expressed Cx, utilizes the clathrin-mediated-endocytosis (CME) pathway, mediated by the clathrin adaptor AP-2, to internalize gap junctions (Piehl et al. 2007, Gumpert et al. 2008, Fong et al. 2013). AP-2 interacts with Cx43 via motifs called tyrosine based sorting signals of which two appear and are used in the Cx43 C-terminus (Fong et al. 2013). In addition, Cx43 is ubiquitinated in a K63-linked pattern, and this ubiquitination is also important for endocytosis (Ribeiro-Rodrigues et al. 2014; Kells and Falk, unpublished). Cx43 is the only Cx whose endocytic mechanism has been studied in detail. Whether the clathrin-mediated internalization mechanism is common or unique throughout the Cx protein family remains to be investigated. It is feasible to speculate that other Cxs utilize other endocytosis strategies or do not undergo endocytosis on their own at all.

We conducted sequence analyses across all Cx subtypes to search for predicted endocytic motifs, that either exhibit known tyrosine (Y-X-X-Φ), or di-leucine (D/E-x-x-x-L-L/I) based sorting signals (Bonifacino & Traub 2003). Using the BLAST search tool we were able to group Cxs into 4 categories, either exhibiting different combinations of the above described canonical sorting signals, or not. Cx family members that do not display canonical endocytic motifs (such as Cx26), may be internalized using an ubiquitin, CLASP- (not AP-2) based pathway. Preliminary studies on Cx26 GJs indicate that they can undergo endocytosis, that they are ubiquitinated, and that they may use an alternative CLASP for endocytosis. Additional functional studies will further examine the role of clathrin and ubiquitin in Cx26 endocytosis and will characterize the endocytosis mechanisms and protein components that may be utilized by other representative Cx family members.

P1450

Board Number: B899

Connexin43 phosphorylation by PKC and MAPK signals gap junction internalization.

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Gap junctions (GJs) are specialized domains in the plasma membrane that consist of clusters of channels that allow direct intercellular communication between neighboring cells. These channels permit ions and small molecules to be transferred from cell-to-cell. Gap junction-mediated intercellular communication (GJIC) is crucial for normal cell function, including coordination of development, tissue homeostasis, growth and differentiation. Moreover, aberrant function of GJs and reduction of cell-cell coupling via GJs has been associated with many pathological conditions, including neuropathies, hearing loss, cataracts, craniofacial anomalies and cancer. GJs exhibit a complex modus of assembly and degradation to maintain balanced intercellular communication. Several growth factors as well as

natural inflammatory mediators, including vascular endothelial growth factor (VEGF), epidermal growth factor (EGF), thrombin and endothelin have been reported to disrupt cell-cell junctions and to abolish GJIC. VEGF directly stimulates VEGF-receptor tyrosine kinases on endothelial cell surfaces. Exposing primary porcine pulmonary artery endothelial cells (PAECs) to VEGF for 15 minutes resulted in a rapid and almost complete loss of connexin43 (Cx43) GJs at cell-cell appositions and a concomitant increase in cytoplasmic, vesicular Cx43. After prolonged incubation periods (60 minutes), Cx43 GJs reformed and intracellular Cx43 restored to levels observed before treatment. These dynamic internalization and relocation events correlated with efficient inhibition of GJIC, up to 3.8-fold increased phosphorylation of Cx43 serine residues 255, 262, 279/282, and 368, and depended on clathrin-mediated endocytosis. Phosphorylation of serines 255, 262, 279/282 was mediated by MAPK, while serine 368-phosphorylation was mediated by PKC. Pharmacological inhibition of both signaling pathways significantly reduced Cx43 phosphorylation and GJ internalization. Together, our results indicate that growth factors, such as VEGF activate a hierarchical kinase program -including MAPK and PKC- that induces GJ internalization via phosphorylation of well-known regulatory amino acid residues located in the Cx43 C-terminal tail.

P1451

Board Number: B900

A novel Panx1-Crmp2 interaction impacts Panx1 surface expression in neural cells.

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Pannexins are four-pass transmembrane proteins that oligomerize to form large-pore channels permeable to ions and metabolites, such as adenosine triphosphate (ATP), of up to 1 kDa. We previously demonstrated that Pannexin 1 (Panx1) promotes neural cell proliferation, at least in part through release of ATP and downstream purinergic receptor signaling. Moreover, we found that Panx1 promotes neural cell migration and inhibits neuronal differentiation. In fact, Panx1 levels are significantly reduced upon neuronal differentiation, and pharmacological block of Panx1 causes dramatic neurite outgrowth. To determine the specific molecular players mediating these roles for Panx1 in neural cells, we performed an unbiased proteomics screen for protein interaction partners using immunoprecipitation coupled to liquid chromatography and tandem mass spectrometry (LC-MS/MS). Here we present data validating and exploring the functional aspects of a specific Panx1-protein interaction with Collapsin Response Mediator Protein 2 (Crmp2). Crmp2 is specifically enriched in the nervous system where it acts as a microtubule binding protein and regulator of growth cone dynamics and neuronal differentiation. We demonstrate that Panx1 and Crmp2 interact both *in vitro*, and *in vivo* in developing and mature neurons. Further, Panx1 preferentially associates with Crmp2 that is un-phosphorylated at the Rho-kinase site, and this interaction appears to be important for Panx1 plasma membrane expression. Finally, using a competitive peptide strategy we find that interfering with the Panx1-Crmp2 interaction alters neural cell behaviors. Overall, we present several novel findings that transform and expand our current understanding of the cellular role of Panx1 in the brain.

P1452**Board Number: B901****Stimulus-evoked internalization of Pannexin 1 channels.**A.K. Boyce¹, L.E. Wicki-Stordeur¹, R.T. Prager¹, M.S. Kim¹, P.C. Nahirney¹, L.A. Swayne¹;¹Division of Medical Sciences, University of Victoria, Victoria, BC

Pannexin 1 (Panx1) is a large pore ion and metabolite permeable channel that is capable of permeating molecules up to 1kDa in size (including ATP). Panx1 has been linked to both neural cell proliferation in the healthy brain and neuronal death in pathological scenarios. To date, exploration of its function has been primarily restricted to its role at the plasma membrane; however, it also localizes to intracellular compartments. We therefore investigated whether the tight regulation of the cell surface versus intracellular Panx1 equilibrium is key for maintaining cell viability. Our first major objective was to definitively characterize the intracellular localization. Initially, confocal microscopy experiments suggested endosomal and nuclear membrane distribution. We confirmed nuclear membrane expression of Panx1 using subcellular fractionation and electron microscopy. We sought to elucidate the mechanism by which Panx1 localizes to these internal membranes because an increase in extracellular ATP causes Panx1 internalization. Interestingly, elevated extracellular ATP inhibits Panx1 activity and ATP is episodically released from neural cells under physiological conditions. Next, to explore retrograde trafficking, we transiently inhibited de novo protein synthesis (and therefore anterograde trafficking) then stimulated with ATP and other stimuli known to modulate Panx1 activity and/or cellular excitability. Using a combination of live and fixed cell confocal imaging, complemented with subcellular fractionation, we show striking evidence for ATP-induced internalization of Panx1 from the plasma membrane with concomitant increase in early endosome and nuclear membrane distribution. We provide additional evidence that the trafficking of Panx1 to the nuclear membranes is critical for cell survival. Our findings suggest that ATP-evoked Panx1 internalization occurs in physiological conditions and would increase in pathological conditions, when extracellular ATP is significantly elevated. ATP-evoked internalization of Panx1 to the nucleus provides an additional mechanism for Panx1 to modulate neural cell proliferation and viability.

P1453**Board Number: B902****Permeability properties of pannexin1 hemichannels activated by mechanical stress.**M. Subiabre¹, C. Puebla¹, A. Vargas¹, P. Fernandez¹, J.C. Saez^{1,2};¹Pontificia Universidad Catolica de Chile, Santiago, Chile, ²Instituto Milenio, CINV, Valparaiso, Valparaiso, Chile

Pannexins (Panx) constitute a protein family with three members (Panx1-3) with little homology to innexins and without homology to connexins. In vertebrates, they are ubiquitously expressed and play

critical roles in numerous physiological and pathological cell responses. Panx1 hemichannels are permeable to ions and small molecules but their permeability features remain controversial and require further characterization. Panx1 hemichannels can be activated via purinergic receptors or membrane stretching. Here, we characterized several features of Panx1 hemichannels. We used HeLa parental cells (HeLa-P) or HeLa cells transfected with Panx1. RT-PCR analyses revealed absence of Panx2 and Panx3. Hemichannels were activated by membrane stretching induced by falling drops of extracellular saline solution. The cell uptake of 4',6-diamidino-2-phenylindol (MW: 350, DAPI(+2)), ethidium (MW: 394, Etd(+)) bromide, propidium iodide (MW: 668, PI(+2)) or Evans blue (MW: 961, EB(-4)) was monitored in time-lapse experiments. Panx1 hemichannels were neither activated by extracellular divalent cations-free solution nor inhibited by La(3+). However, they were activated by mechanical stress and inhibited by carbenoxolone (5 μ M). The uptake showed a Michaelis-Menten behavior with characteristic K_m for each dye. HeLa-Panx1 cells did not present PI(2+) uptake and uptake of all dyes was absent in HeLa-P cells. Therefore, Panx1 hemichannels present low permeability to cationic molecules and are highly permeable to anionic molecules.

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P1454

Board Number: B903

Complex Mechanisms Regulate the Gap Junction Coupling of Blood-Brain Barrier Endothelial Cells by Adenosine Receptor Stimulation.

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The human cerebral microvascular endothelial cell line hCMEC/D3 is an accepted *in vitro* model of the endothelial cells of the blood-brain barrier (BBB). Recent data have shown that regulation of gap junction coupling in cerebral endothelial cells could be related to blood-brain barrier function. In our project, PCR experiments showed that the hCMEC/D3 cells expressed the adenosine receptor subtypes A₁, A_{2A} and A_{2B} as well as connexin37 (Cx37), Cx40, Cx43 and Cx45. The adenosine receptor agonist 2-phenylaminoadenosine (2-PAA) stimulated synthesis of cAMP in hCMEC/D3 cells, indicating that the receptor molecules were present in the cell membrane. Additionally, 2-PAA enhanced the gap junction coupling as shown by scrape loading/dye transfer experiments. The diffusion distance of Lucifer Yellow in hCMEC/D3 cells was increased from 66 μ m to 87 μ m by 5 μ M 2-PAA applied for 1 h. The increase achieved a maximum of 95 μ m after application of 20 μ M 2-PAA. The inhibitor of the A_{2B} adenosine receptor subtype, MRS1754 reduced the 2-PAA-related enhancement of the gap junction coupling back to control levels, whereas the inhibitor of the A_{2A} receptor subtype, SCH58261 could not significantly suppress the effect of 2-PAA on gap junction coupling. This indicates that the A_{2B} receptor subtype is predominantly involved in the regulation of gap junction coupling of the hCMEC/D3 cells. The inhibitor of the protein kinase A (PKA), Rp-cAMPS did not affect the 2-PAA-induced increase in gap junction

coupling while the activator of Epac, 8-pCPT-2'-O-Me-cAMP could not increase the gap junction coupling in the hCMEC/D3 cells. Interestingly, the 2-PAA-related enhancement of the gap junction coupling was significantly reduced if the drug was applied in absence of external Ca^{2+} or to cells preloaded with the Ca^{2+} chelator BAPTA. The results suggest that even if cAMP is produced by the stimulation of adenosine receptors in cerebral microvascular endothelial cells, the action on gap junction coupling does not only involve cAMP-activated proteins such as PKA or Epac but Ca^{2+} -dependent pathways are activated as well.

P1455

Board Number: B904

Distinct nano-scale organization of paired receptors at Natural Killer cell surfaces revealed by super-resolution microscopy.

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Human Natural Killer (NK) cells are regulated by a variety of germ-line encoded activating and inhibitory receptors. Broadly, activating receptors detect ligands that are expressed or up-regulated on cancerous or infected cells, while inhibitory receptors bind self-molecules to induce tolerance against healthy cells. Highly homologous pairs of activating and inhibitory receptors are also expressed on NK cells, including Killer Ig-like Receptors KIR2DL1 and KIR2DS1, which bind the same ligands, class I MHC proteins. With a view to understanding signal integration between these paired receptors, we used two super-resolution microscopy techniques - Ground State Depletion (GSD) and Stimulated Emission Depletion (STED) microscopy - to determine how KIR2DL1 and KIR2DS1 are organized on a nanometre-scale. Firstly, neither receptor was distributed randomly; both were observed to constitutively assemble in nanometre-scale clusters. $39 \pm 13\%$ of KIR2DS1 molecules were found in clusters, distributed at 3.5 ± 0.7 clusters/ μm^2 , while only $27 \pm 9\%$ of KIR2DL1 accumulated in clusters, at 8 ± 2 clusters/ μm^2 . Importantly, the activating receptor KIR2DS1 formed nano-clusters 2.4-fold larger (diameter 120 ± 13 nm) than its inhibitory counterpart KIR2DL1. The density of KIR2DS1 and KIR2DL1 within clusters was 7.5 and 4.5 fold greater than the average membrane density, respectively. Interestingly, clusters of KIR2DL1 and KIR2DS1 were significantly segregated from each other (with a negative Pearson's correlation coefficient). Thus, these data establish that paired NK cell receptors have a distinct nano-metre scale organisation at the cell surface which likely influences signalling; their segregation rules out models of signal integration which requires paired receptors to be juxtaposed to each other.

P1456**Board Number: B905****Triggering receptor expressed on myeloid cells1 (TREM-1) and innate immune responses to Plasmodium falciparum.**S. ADUKPO^{1,2}, P.G. KREMSNER², V.P. Thirumalaisamy²;¹IMMUNOLOGY, NOGUCHI MEMORIAL INSTITUTE FOR MEDICAL RESEARCH, ACCRA, Ghana, ²INSTITUTE FOR TROPICAL MEDICINE, UNIVERSITY OF TUEBINGEN, TUEBINGEN, Germany

Monocytes and Neutrophils mediate anti-malarial immune responses and release soluble factors including reactive oxygen species (ROS) that are toxic to the parasite. These inflammatory responses could be enhanced or dampened when certain receptors on immune cells are activated. Triggering receptor expressed on myeloid cells-1 (TREM-1) is a recently described trans-membrane and immune-regulatory receptor strongly expressed on neutrophils and in a subset of monocytes and macrophages. TREM-1 plays a central role in regulating innate immunity as it amplifies the inflammatory signals initiated by pathogen associated molecular pattern recognition receptors (PAMPs) such as Toll-like (TLR) and NACHT-LRRs. TREM-1 is of functional importance in a number of inflammatory diseases. Till-date, the involvement of TREM-1 in malaria pathogenesis is not investigated. We analyzed the effect of TREM-1 signaling on the responsiveness of Monocytes and Neutrophils to crude Plasmodium falciparum antigens and live parasites in the presence or absence of TREM-1 inhibitor. We co-incubated neutrophils and PBMCs with malaria antigens and subsequently measured ROS production over time by Chemiluminescence. Parasitaemia and parasite growth inhibition were also evaluated. Significant inhibition of ROS production was observed when TREM-1 was blocked. However no significant contribution to inhibition of P. falciparum growth was observed. In conclusion, TREM-1 was activated during interaction between Monocyte/Neutrophils and parasite but its activation does not seem to affect the growth of the P. falciparum in vitro.

P1457**Board Number: B906****Dysregulation of nectin-2 in the testicular cells: an explanation of cadmium-induced male infertility.**W. Lui¹, X. Zhang¹;¹School of Biological Sciences, University of Hong Kong, Hong Kong, Hong Kong

Nectin-2, a junction molecule, is found at the basal and apical ectoplasmic specializations (ES) for the formation of the blood-testis barrier (BTB) (constituted by tight junctions and basal ES) and Sertoli-spermatid adhesion. Loss of nectin-2 causes male infertility, suggesting nectin-2-based ES is crucial for spermatogenesis. Cadmium (Cd) has been known to induce severe testicular injury. Recent evidence has shown that the basal ES at the BTB and apical ES are the targets of Cd, suggesting that unique junction protein at the ES may explain why the testis is more susceptible than other tissues. Since nectin-2 is

expressed exclusively at the ES, it is highly possible that nectin-2 is the direct target of Cd. In this study, we investigate if nectin-2 is the target protein of Cd toxicity and the mechanism on how Cd down-regulates nectin-2 to achieve ES disruption. Our results revealed that Cd suppresses nectin-2 at transcriptional and post-translational levels. Inhibitor and shRNA knockdown have shown that Cd induces nectin-2 protein degradation via clathrin-dependent endocytosis. Immunofluorescence staining and endocytosis assays further confirmed that nectin-2 internalization is promoted upon Cd treatment. Besides, Cd directly represses nectin-2 transcription. EMSA and ChIP assays showed that Cd inhibits the binding of positive regulators to nectin-2 promoter. siRNA and overexpression analyses have demonstrated that Cd reduces the expression and binding affinity of positive regulators for transcription. Taken together, nectin-2 is the direct molecular target of Cd and its disruptive effects is mediated via direct repressing nectin-2 transcription and inducing endocytosis of nectin-2 for degradation.

P1458

Board Number: B907

High levels of glucose modulate tight junction-associated epithelial barrier function of renal tubular cell line: possible implication in diabetic nephropathy.

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Type 2 diabetes mellitus (T2DM) is one of the most prevalent diseases worldwide. Diabetic nephropathy (DN) is a complication of diabetes and the mechanisms associated with the onset and progression of this disease is not fully understood. It has been shown that hyperglycemia is an independent factor to predict the development of DN in individuals with T2DM, however the mechanisms involved in kidney injuries associated with high plasma glucose levels remain unknown. In the present study, we investigated the in vitro effect of high levels of glucose (i.e. 180 or 360mg/dL) up to 24h upon epithelial barrier integrity of the kidney tubular cell line, MDCK. High levels of glucose (180 and 360 mg/dL) induced a tendency of decrease in transepithelial electrical resistance (TER) associated with subtle increase in epithelial paracellular permeability, as assessed by phenol red transepithelial flux, when compared to the control group (exposed to 100mg/dL glucose). Immunofluorescence analyses showed that glucose treatment induced a significant decrease in the tight junctional content of Claudin-1 as well as a significant increase in Claudin-2 and Occludin. The analyses of cell content of these junctional proteins by Western Blot corroborate the immunofluorescence data. Our data suggest that high levels of glucose induce changes in TJ structure in MDCK monolayers, suggesting a possible link between hyperglycemia-induced tubular epithelial barrier disruption and diabetic nephropathy. Financial support: FAPESP/Brazil (#2013/15767-0).

P1459**Board Number: B908****Towards identifying the mechanism by which Anillin localizes to cell-cell junctions and regulates junction integrity.**C.C. Reyes¹, A.L. Miller²;¹Program in Cellular and Molecular Biology, University of Michigan, Ann Arbor, MI, ²Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI

Anillin is a scaffolding protein that organizes and stabilizes actomyosin contractile rings and was previously thought to function primarily in cytokinesis. Using *Xenopus laevis* embryos as a model system to examine Anillin's role in the intact vertebrate epithelium, we recently identified a novel role for Anillin in regulating epithelial cell-cell junctions¹. We found that a population of Anillin surprisingly localizes to epithelial cell-cell junctions throughout the cell cycle where it plays a critical role in regulating cell-cell junction integrity. Both tight junctions and adherens junctions are disrupted when Anillin is knocked down, leading to abnormal intercellular spaces and increased permeability to fluorescent dextran. Anillin was known to interact with RhoA, F-actin, and Myosin II, all of which regulate cell-cell junction structure and function. Junctional F-actin and Myosin II accumulation are reduced when Anillin is knocked down and increased when Anillin is overexpressed. Additionally, increased dynamic "flares" of RhoA-GTP are observed at cell-cell junctions when Anillin is knocked down. We propose that Anillin is required for proper RhoA-GTP distribution at cell-cell junctions and for maintenance of a robust apical actomyosin belt, which are required for cell-cell junction integrity. Current work seeks to determine where Anillin localizes with respect to tight junctions and adherens junctions, how Anillin is targeted to cell-cell junctions, and which domains of Anillin are responsible for proper regulation of cell-cell junction structure and function.

¹Reyes, C.C., Jin, M., Breznau, E.B., Espino, R, Delgado-Gonzalo, R., Goryachev, A.B., **Miller, A.L.** (2014). Anillin Regulates Cell-Cell Junction Integrity by Organizing Junctional Accumulation of Rho-GTP and Actomyosin. *Current Biology*. 24:1263-1270.

Focal Adhesions and Invadosomes**P1460****Board Number: B909****Fetal Fibroblasts Exhibit Altered Mechanosensing Via Reduced Adhesion Formation and Force Production on Rigid Substrates.**A. Parekh¹, R. Jerrell¹;¹Department of Otolaryngology, Vanderbilt University Medical Center, Nashville, TN

In post-natal or "adult" dermal wound healing, fibroblasts mechanically sense ("mechanosense") rigidity and tension that develop in granulation tissue and differentiate into myofibroblasts. Myofibroblasts

generate large contractile forces that excessively contract and remodel the ECM resulting in scarring and fibrosis. In contrast, injured skin in the mammalian fetus heals scarlessly without myofibroblast involvement suggesting that smaller cellular forces contribute to regenerative repair. We have previously shown that populations of human dermal fetal fibroblasts significantly contract mechanically stressed type I collagen gels to a lesser extent than adult fibroblasts and exhibit less alpha-smooth muscle actin-rich stress fibers. These results suggest that fetal fibroblasts exhibit differential responses to matrix rigidity which normally induce myofibroblast differentiation in adult fibroblasts. Therefore, we hypothesized that fetal fibroblasts have intrinsically altered mechanosensing. Using polyacrylamide gels that mimic the different mechanical stages of wound healing that progressively induce myofibroblast differentiation, we tested whether a defect existed in focal adhesion maturation and contractile force generation in fetal fibroblasts in response to matrix rigidity. In comparison to adult fibroblasts, we found that initial adhesion formation and maturation in fetal fibroblasts after one hour is impaired on polyacrylamide gels that correspond to the rigidity of late stage granulation tissue. We also found a reduction in contractile forces exerted by fetal fibroblasts after overnight incubation on the same polyacrylamide gels. Therefore, our data suggest that fetal fibroblasts have altered mechanical responses to physiologic rigidities which may contribute to their lack of myofibroblast differentiation leading to scarless dermal healing.

P1461

Board Number: B910

Distribution of vinculin to lipid rafts is involved in sensing ECM stiffness.

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Extracellular matrix (ECM) is a key component of extracellular microenvironments, which are important for cell migration, proliferation and differentiation. In addition, ECM stiffness acts as a regulator of mesenchymal stem cell differentiation and lineage selection. Cells sense ECM stiffness through mesoscopic scale cell adhesion complexes, called focal adhesions (FAs). FAs consist of ECM receptors, integrins, which are connected to the ends of actin stress fibers through various cytoplasmic proteins, including talin and vinculin. Recently, our group has shown that interaction of vinculin with vinexin α , both of which are cytoplasmic FA proteins, play a critical role in sensing ECM stiffness and act as a mechanosensor in mouse embryonic fibroblasts (MEFs). Lipid rafts are membrane mesoscopic-domains where phosphatidylinositol 4,5-bisphosphate (PIP₂) and cholesterol are enriched. They have been implicated in diverse cellular processes including signal transduction. Here we investigated whether lipid rafts are involved in sensing ECM stiffness. First, we examined the distribution of vinculin to lipid rafts. Membrane fractions were extracted from WT MEFs and treated with 1% TritonX-100 at 4°C, followed by density-gradient ultracentrifugation. Certain part of vinculin and vinexin α were separated into the fractions containing flotillin, a raft maker protein. Furthermore, depletion of vinexin expression impaired distribution of vinculin to the raft fraction. Re-expression of vinexin α rescued the vinculin distribution,

indicating that vinculin α is necessary for vinculin distribution to the raft fraction. In addition, Mutation in the binding site for vinculin α also impaired the distribution of vinculin to the raft fraction, indicating that an interaction of vinculin with vinculin α is required for the distribution of vinculin to lipid rafts. Next we tested the effect of intracellular tension and ECM stiffness on the distribution of vinculin. Reducing intracellular tension by the treatment with blebbistatin, a myosin II inhibitor, decreased the distribution of vinculin to the raft fraction. Culturing on rigid ECM promoted the distribution of vinculin to the raft fraction compared to the culturing on soft ECM. These results suggest that intracellular tension and ECM stiffness are involved in the distribution of vinculin to lipid rafts. We previously reported that residence time of vinculin at FAs is important for sensing ECM stiffness. Thus we examined the effect of raft disruption on residence time of vinculin at FAs. Treatment with rafts-disrupting reagent (Methyl-beta-cyclodextrin) decreased the residence time of vinculin at FAs on rigid ECM. Taken together, these results suggest that distribution of vinculin to lipid rafts is involved in sensing ECM stiffness.

P1462

Board Number: B911

Fluctuation in Single Molecule Talin Stretching Correlates with Vinculin Binding in vivo.

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Talin molecules in focal adhesions (FA) connect integrin to the actin network and recruit other FA proteins. They transmit and sense the forces between the extracellular matrix (ECM) and the cytoskeleton possibly by means of their cyclic stretching behavior in live cells. The stretching of an individual talin molecule exposes vinculin-binding sites on its rod domain and thereby welcomes the binding of vinculin which in consequence enhances the connection strength of the actin network to integrins. In a continuation of our previous study, employing multi-channel multi-molecule and single-molecule tracking techniques, we further explored the dynamic stretching behaviour of talin and its quantitative relationship to vinculin recruitment. Both the N terminus and C terminus of talin are cyclicly moving in a stretching event; the N terminus movement is due to the – possibly random - walk of integrin molecules and the C terminus movement is directly coupled to the actin flow. The talin stretching event correlates with vinculin recruitment both on the single molecule level and the photometry observation level. During a single talin dimer-stretching event in live cells, the method reported up to 12 vinculin molecules being recruited. With most of the vinculin binding events occurring in close proximity of the C terminus rather than the N terminus between the termini. In summary, the talin stretching recruitment events are correlated to the vinculin level in focal adhesions and multiple vinculins can bind to a single talin in the same cycle in live cells.

P1463**Board Number: B912****iPALM and FRET reveal the mechanism of vinculin activation and nano-scale spatial organization in focal adhesions.**L. Case¹, M. Baird², G. Shtengel³, S. Campbell⁴, H. Hess³, M.W. Davidson⁵, C. Waterman⁶;¹Cell Biology and Physiology Center, NHLBI/NIH, Bethesda, MD, ²Florida State University, Tallahassee, FL,³Janelia Farm, HHMI, Ashburn, VA, ⁴Biochemistry and Biophysics, UNC, Chapel Hill, NC, ⁵National High Magnetic Field Laboratory and Department of Biological Science, Florida State University, Tallahassee, FL, ⁶National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD

Focal adhesions (FAs) are multi-protein complexes that link the extracellular matrix (ECM) to the actin cytoskeleton to mediate cell adhesion, migration, mechanosensing and signaling. Previously, interferometric photoactivatable localization microscopy (“iPALM”), which uses single molecule localization to measure lateral position with ~20nm accuracy and interferometry to measure the axial position with ~10nm accuracy, revealed that FAs have a conserved laminar structure, with an integrin signaling layer (ISL) ~10-20 nm from the plasma membrane, an actin regulatory layer (ARL) ~100 nm from the membrane that extends into the stress fiber, and a force transduction layer (FTL) that spans the intervening space. However, it is unknown how the layered organization of proteins within focal adhesions contributes to the regulation of protein activity and function. Vinculin (Vcl) is a protein that undergoes a conformational change at focal adhesions to interact with multiple binding partners and regulate diverse cellular functions including protrusion, migration, mechanosensing and survival. Vcl has over 10 binding partners distributed throughout the FA including paxillin in the ISL, talin (Tln) in the FTL, and actin in the ARL. We hypothesized that the spatial compartmentalization of different binding partners into distinct FA layers contributes to Vcl activation, regulation and functional specificity at focal adhesions. To test this, we used point mutants to perturb specific protein interactions and assayed Vcl nanoscale localization and Vcl activation state at FAs. We used iPALM to image the 3D localization of vinculin molecules with nanometer accuracy as well as a FRET biosensor to assay vinculin conformational changes. By systematically perturbing specific protein interactions, we found that vinculin is recruited to the ISL in an inactive conformation by its interaction with paxillin. Subsequently, vinculin activation is mediated by talin and actin binding, and talin binding promotes vinculin association with the higher FTL and ARL. Active vinculin localizes ~10nm higher than inactive vinculin in FA. Vcl exhibits a gradient of low-to-high activation from the front-to-back of FA, and Vcl exhibits increased localization with the ARL in the back of FA. Finally, while Vcl is not required for the proper nanoscale localization of paxillin or actin, Vcl is required for Tln to be maintained in the maximally vertically extended conformation. By performing complimentary experiments with iPALM and FRET, we have been able to study the mechanism of vinculin activation on a structural level in a cellular setting. We propose that movement up the laminar FA structure facilitates Vcl activation and mechanical reinforcement of FA.

P1464**Board Number: B913****Role of Talin in Determining the Nanoscale Architecture of Focal Adhesions.**J. Liu¹, Y. Wang¹, W. Goh¹, H. Goh¹, M. Baird², M.W. Davidson³, P. Kanchanawong^{1,4};¹Mechanobiology Institute, Singapore, Singapore, ²Florida State University, Tallahassee, FL, ³National High Magnetic Field Laboratory and Department of Biological Science, Florida State University, Tallahassee, FL, ⁴National University of Singapore, Department of Biomedical Engineering, Singapore, Singapore

Focal adhesions (FAs) are integrin-based structures that mediate cell-extracellular matrix interactions, playing central roles in signaling, cell motility, and mechanotransduction. Although FAs has commonly been described and modeled as molecular machines, the interplay of ultrastructural, biophysical, and biochemical factors that underlie their multifunctional roles is still not well understood. Earlier, using superresolution microscopy we described the nanoscale architecture of FAs, in which several key proteins are stratified into nanoscale layers interposing between the integrin cytoplasmic tails and the actin cytoskeleton (Kanchanawong et al., Nature 2010). However, the molecular basis of how such nanoscale organization emerges remains poorly understood. Based on our prior observation that talin appears to be oriented in a polarized manner in FAs, with the N-terminus in the membrane-proximal zone and the C-terminus at the FA core/actin stress fiber interface, we hypothesize that talin may serve to regulate FA nanostructures. To test this, we constructed a series of talin analogues with lengths modified by partial deletions in the central rod domains, and performed superresolution imaging in conjunction with siRNA-mediated substitution of endogenous talin by such talin analogues. By probing the position of the N- or C- termini using fluorescent protein tags, we observed that the N-terminal position remains largely constant, while the C-terminal positions shifted downward in proportion to the molecular dimension, indicating that the talin analogues adopted an oriented organization similar to the full-length talin. Corresponding downshifts in the positions of the FA core/ actin stress fiber interface were also observed, suggesting that the FA core dimension may be controlled by the molecular dimension of the talin analogues. A lengthening modification by the insertion of a ~30-nm long FilaminaA segment resulted in the proportional upshifts of the C-terminal positions of the talin analogues in FAs, suggesting that talin modulates FA core dimension in a highly modular manner. Altogether, our results demonstrated how talin serves to determine the nanoscale architecture underlying the FA machinery through its molecular dimension, akin to a molecular ruler.

P1465**Board Number: B914****GEF-H1 controls focal adhesion signaling that regulates mesenchymal stem cell lineage commitment.**J. Kuo¹, I. Huang², C. Hsiao^{3,4}, J. Wu⁵, R. Shen⁶, C. Liu^{7,8}, Y. Wang^{9,10}, Y. Chen⁵, C. Huang⁵, J. Del Alamo¹¹, Z. Chang⁵, M. Tang¹², K. Khoo^{3,4};¹Institute of Biochemistry and Molecular Biology, National Yang Ming University, Taipei, Taiwan,²National Yang Ming University, Taipei, Taiwan, ³Institute of Biochemical Sciences, National TaiwanUniversity, Taipei, Taiwan, ⁴Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan, ⁵TheInstitute of Biochemistry and Molecular Biology, National Yang Ming University, Taipei, Taiwan, ⁶Centerfor Biologics Evaluation and Research, U.S. Food and Drug Administration, Bethesda, MD, ⁷Institute ofBasic Medical Sciences, National Cheng Kung University Hospital, Tainan, Taiwan, ⁸Department ofPhysiology, National Cheng Kung University Hospital, Tainan, Taiwan, ⁹Department of Cell Biology andAnatomy, National Cheng Kung University Hospital, Tainan, Taiwan, ¹⁰Center for Neurotrauma andNeuroregeneration, Taipei Medical University, Taipei, Taiwan, ¹¹University of California, San Diego, LaJolla, CA, ¹²Natl Cheng Kung Univ Med Coll, Tainan, Taiwan

Focal adhesions (FAs) undergo maturation culminating in size and composition changes that modulate adhesion, cytoskeleton remodeling and differentiation. While it is well-recognized that stimuli for osteogenesis of mesenchymal stem cells (MSCs) drive FA maturation, actin organization, and stress-fiber polarization, the extent to which FA-mediated signals regulated by the FA protein composition specifies MSC commitment remains largely unknown. Here we demonstrate that, upon dexamethasone (osteogenic induction) treatment, guanine nucleotide exchange factor-H1 (GEF-H1) is significantly enriched in FAs. Perturbation of GEF-H1 inhibits FA formation, anisotropic stress-fiber orientation and MSC osteogenesis in an actomyosin contractility-independent manner. To determine the role of GEF-H1 in MSC osteogenesis, we explore the GEF-H1-modulated FA proteome that reveals non-muscle myosin-II heavy chain-B (NMIIB) as a target of GEF-H1 in FAs. Inhibition of targeting NMIIB into FAs suppresses FA formation, stress-fiber polarization, cell stiffness and osteogenic commitments in MSCs. Our data demonstrate FA signaling in specifying MSC commitment.

P1466**Board Number: B915****Autophagy regulates focal adhesion turnover in transformed cells.**C.M. Kenific¹, S. Stehbens², K. Woo¹, J. Ye¹, T. Wittmann³, J. Debnath¹;¹Department of Pathology, University of California, San Francisco, San Francisco, CA, ²QueenslandUniversity of Technology, Queensland, Australia, ³Department of Cell and Tissue Biology, University of

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Autophagy is a process of lysosomal-mediated degradation that has traditionally been viewed as a stress-adaptation pathway that promotes tumor cell survival. However, recent evidence suggests that

autophagy serves broader functions during cancer progression. Here, we demonstrate a new role for autophagy in regulating focal adhesion (FA) turnover in transformed cells.

Autophagy inhibition via RNAi-mediated depletion of essential autophagy regulators (ATGs) decreases single-cell migratory rate of cells in a wound healing assay, and wound edge cells have significantly enlarged FAs upon autophagy inhibition. Because FA assembly and disassembly, or turnover, regulates migratory rate we hypothesized that increased FA size was due to impaired FA turnover. Using spinning disk confocal (SDC) microscopy to image FA dynamics in live cells expressing Paxillin-mCherry, to mark FAs, we observed that ATG depletion decreased FA assembly and disassembly rates, while increasing FA lifetime, suggesting that stabilization of FAs upon autophagy inhibition impairs motility. To further analyze the relationship between autophagy and FAs, we determined if autophagosomes localize near FAs. Total internal reflection fluorescence microscopy of live cells revealed that autophagosomes, as marked by GFP-LC3, are found in proximity to FAs. Furthermore, SDC imaging showed that autophagosomes associate with dynamic FAs primarily during the disassembly phase of the turnover cycle.

Because autophagy is pathway of cellular degradation, these findings led us to hypothesize that autophagy facilitates FA disassembly by locally degrading FA components. To test this prediction, we focused on establishing if autophagy cargo receptors, which mediate selective autophagic degradation of cellular substrates by linking cargo to autophagosomes, modulate FA turnover. We found that similar to ATG depletion, knockdown of the cargo receptor NBR1 impairs FA turnover. Conversely, overexpression of NBR1 decreases FA lifetime, and GFP-NBR1 associates with dynamic FAs. These data suggest that NBR1-mediated selective autophagy controls FAs in transformed cells. We are now testing this model more rigorously using autophagy-defective mutants of NBR1.

In addition to regulating motility, cell-matrix adhesions also promote growth signals that drive metastatic colonization. Thus, enhanced cell-matrix adhesion due to increased FAs upon autophagy inhibition may support metastasis. Accordingly, ATG depletion in a polyoma middle T (PyMT) breast carcinoma cell line leads to enlarged FAs *in vitro* and enhanced pulmonary metastasis *in vivo*. We are now determining if increased cell-matrix adhesion is a mechanism by which autophagy inhibition potentiates metastasis and if NBR1 regulates metastasis of PyMT cells.

P1467

Board Number: B916

Differential Mechanisms of Matrix Degradation by Tumor Cells Versus Stromal Fibroblasts.

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Metastatic invasion of tumors into peripheral tissues is known to rely upon protease-mediated degradation of the surrounding stroma. This remodeling process utilizes complex, actin-based,

specializations of the plasma membrane (PM) termed “invadopodia” that act to both sequester and release matrix metalloproteases. Two well-established components of invadopodia are the large membrane remodeling GTPase Dynamin 2 (Dyn 2), and oncogenic Src kinase. Here we report that cells of mesenchymal origin degrade substantial amounts of surrounding matrix by a mechanism, in part, independent of conventional invadopodia. Two distinct human, or rat, cultured fibroblast cell lines (HFs/RFs) or a pancreatic tumor stellate cell line (PSC) exhibit little to no matrix degradation when plated on fluorescent gelatin over a 24 hour period. Surprisingly, siRNA mediated knock-down of Dyn 2 in these cells induces a massive increase (3-4 fold) of substrate degradation. These degradative sites lack the punctate shape of conventional invadopodia but are instead spread along the cell base and are reticular and/or fibrous in character. In strong support of these findings, a tamoxifen activated knock-out of Dyn 2 in mouse embryonic fibroblasts also induces a marked increase (7-8 fold) in the degradation of matrix exhibited by these cells. Re-expression of wild type Dyn 2 or K44A GTPase defective mutant, actually reduces/eliminates matrix degradation to near normal levels. In strong contrast, siRNA knock down of Dyn 2, or expression of Dyn 2 K44A, in pancreatic adenocarcinoma cell lines (DanG, BxPC3) resulted in a near complete inhibition of canonical invadopodial-based degradation consistent with past findings. As matrix degradation in the mesenchymal cell lines appears to be induced by a reduction in Dyn 2 levels and exhibits a pattern distinct from conventional invadopodia, we tested a role for Src kinase in this novel process. While treatment of the pancreatic tumor cells with PP2 or SU6656 inhibitors markedly reduced invadopodia-based matrix degradation these had almost no effect on this process in the fibroblast cell lines. These findings provide evidence for a novel matrix remodeling process conducted by mesenchymal cells that is substantially more effective than conventional invadopodia, distinct in structural organization, and Dyn 2 and Src kinase independent.

P1468

Board Number: B917

Extracellular matrix compliance and Rho-family GTPase signaling guide plasticity of tumor cell invasion.

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Tumor cell invasion is a complex process that requires the molecular and physical adaptation of both the cell and its microenvironment. Increased MAPK signaling, small GTPase activation, cytoskeletal rearrangements, and the directed targeting of proteases to sites of extracellular matrix (ECM) degradation all accompany the process of tumor cell invasion. We have shown that nucleotide cycling on the ARF6 protein regulates the release of protease-loaded, plasma membrane-derived microvesicles from tumor cells into the microenvironment to promote ECM degradation. These shed vesicles are distinct from invadopodia, which are protrusions at the adherent surface of cells and mediate cell invasion. While the release of protease-loaded microvesicles potentially serves as a mechanism to bring about matrix degradation and also deposit paracrine information at distal locations creating paths of least resistance, invadopodia facilitate pericellular proteolysis of the ECM at the cell's invasive front and

appears to be linked to mesenchymal type tumor cell motility. We show that Rho GTPase signaling and the matrix microenvironment govern whether tumor cells produce invadopodia, or adopt an amoeboid phenotype and release microvesicles. The ability of tumor cells to switch between the aforementioned, qualitatively distinct modes of invasion may facilitate movement through different microenvironments. Understanding the interplay between these two invasive cell programs will provide new and important insights into the progression toward pathophysiological states.

P1469

Board Number: B918

Macrophage infiltration of the stroma in benign prostatic hyperplasia (BPH).

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Benign prostatic hyperplasia (BPH) has been previously characterized by inflammation and the increased presence of macrophages. Here, we seek to identify factors that mediate macrophage invasion in BPH. To test macrophage invasion *in vitro*, we embedded macrophage-differentiated THP-1 cells into a three-dimensional (3D) fibrin matrix and measured podosome formation. Using this model, we found that exogenous, but not endogenous, fibronectin was important for macrophage podosome formation. Supporting the clinical relevance of this mechanism, the prostatic stroma is rich in fibronectin and macrophages were found to co-localize with fibronectin in the stroma. Interestingly, a higher percentage of macrophages were found in the stroma of BPH prostate compared to matched normal prostate samples. Furthermore, conditioned medium from prostatic stromal cells promoted macrophage invasion in fibrin-fibronectin matrices. Together, these results suggest an important role for the stroma in promoting macrophage infiltration of BPH tissue. Ongoing work seeks to identify the mechanism by which stromal cells stimulate macrophage invasion.

P1470

Board Number: B919

Two distinct actin networks mediate traction oscillations to confer mechanosensitivity of focal adhesions.

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Adherent cells actively sense the mechanical stiffness of their extracellular matrix (ECM) by exerting traction force through focal adhesions (FAs), which are integrin-based protein assemblies. Also, FAs control cell spreading, proliferation, survival, differentiation, and migration. FA-mediated

mechanosensation underlies cell durotaxis – the tendency of most cell types to migrate toward stiffer microenvironment. Strikingly, FA-mediated traction forces oscillate in time and space, and this oscillation governs durotaxis. The interactions underlying this intriguing spatio-temporal pattern of FA traction force are unknown, as are the contributions of these interactions to this mechanosensation. To address these questions, we established the first coherent, experimentally validated model of FA formation. The model integrated the spatiotemporal coordination between a branched actin network and stress fibers during FA growth. Our model predicted that retrograde flux of branched actin network contributed to a traction peak near the FA distal tip and that stress fiber-mediated actomyosin contractility generated a second traction peak near the FA center. Furthermore, a negative feedback loop involving formin-mediated stress fiber elongation and actomyosin contractility developed and resulted in oscillation of the center traction peak. This oscillation competed with the distal traction peak, and the competition underpinned oscillation of the FA traction maximum in time and space. More importantly, this negative feedback loop broadened the substrate stiffness range, over which the FAs could accurately adapt with traction force generation. Our findings shed light on the fundamental mechanism of FA mechanosensation and durotaxis.

P1471

Board Number: B920

Rigidity Signals Differentially Regulates Invadopodia Activity Through the ROCK Isoforms.

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Rho-associated kinase (ROCK) activity increases due to extracellular matrix (ECM) rigidity in the tumor microenvironment and drives cancer cell migration and invasion via actomyosin contractility. Invasion through cross-linked tissues is facilitated by actin-rich adhesive protrusions called invadopodia that proteolytically degrade the ECM. We have previously shown that ECM degradation by invadopodia is dependent on matrix rigidity and traction forces generated by non-muscle myosin II (NM II) suggesting that invadopodia activity is regulated by rigidity signals transmitted through cellular contractility. However, ROCK exists in two isoforms that may participate in other signaling pathways to regulate invadopodia activity independent of force generation. In particular, the ROCK substrate LIM kinase (LIMK) has been shown to regulate invadopodia activity to drive cancer cell invasion. Therefore, we hypothesized that rigidity signals induce differential ROCK activity to regulate invadopodia. Using siRNA to specifically inhibit each ROCK isoform, we investigated the contractile and invasive responses of an aggressive head and neck carcinoma cell line using polyacrylamide gels of different rigidities that span reported values for the mechanical properties of increasing grades of human tumor tissues. We found that only ROCK1 inhibition decreased traction forces while both ROCK1 and ROCK2 inhibition decreased ECM degradation. We also found that downstream ROCK signaling through both NM II and LIMK was dependent on matrix rigidity and regulated by ROCK1 and ROCK2, respectively. Therefore, our data suggest that matrix rigidity activates differential ROCK activity through two non-redundant signaling

pathways that converge to regulate invadopodia activity and may contribute to invasive behavior by cancer cells in response to the mechanical properties of the tumor microenvironment.

P1472

Board Number: B921

Laminin-derived peptide C16 regulates migration, invasion, invadopodia key molecules, and ROS generation in human prostate cancer cells.

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Prostate cancer is the second most frequently diagnosed cancer and sixth leading cause of cancer death in males. During tumor growth, neoplastic cells interact with the extracellular matrix (ECM) and this interaction influences tumor biology. Laminin is a molecule prominently expressed in ECM, and a key regulator of different tumors. Our Laboratory has demonstrated that peptides derived from laminin-111 cleavage are involved in migration, invasion and invadopodia formation in different tumor cell lines. Invadopodia are membrane protrusions made up by actin and actin-binding proteins. Invadopodia activity depends on expression of the proteins Tks4, Tks5, cortactin, MT1-MMP, as well as reactive oxygen species (ROS) generation. Here we analyzed the effect of C16, a laminin-derived peptide, on migration, invasion and invadopodia activity in human prostate cancer cells (DU145). Migration assays in chemotaxis chambers demonstrated that C16 increased migration activity of DU145 cells in a dose-dependent manner compared to cells grown on C16SX (scrambled peptide control). C16 also increased invasion of DU145 cells in a dose-dependent manner. We addressed signaling pathways related to C16 effects in tumor cells, and observed that the peptide stimulated Src phosphorylation. Migration and invasion are related to protease expression and invadopodia activity. Fluorescent substrate degradation assay showed that C16 increased invadopodia activity of DU145 cells. Immunoblot revealed that this peptide stimulated expression of key invadopodia molecules, such as Tks4, Tks5, cortactin and MT1-MMP. Reactive oxygen species (ROS) generation is also related with invadopodia formation. This prompted us to address whether C16 would induce ROS generation in DU145 cells. To detect ROS, control and treated cells were loaded with CellROX Deep Red kit. Quantitative fluorescence and flow cytometry showed that the peptide C16 increased ROS in DU145 cells. Our results strongly suggested that laminin peptide C16 regulates migration, invasion, invadopodia key molecules, and ROS generation in human prostate cancer cells (DU145). Support: FAPESP (2008/57103-8, 2009/17336-6, 2009/16150-6) and CNPq (304986/2009-7)

P1473**Board Number: B922****Mena localizes to invadopodia through interactions with specific targeting proteins to promote matrix degradation in breast carcinoma cells.**M.D. Weidmann¹, V.P. Sharma^{1,2}, J.S. Condeelis¹;¹Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY, ²Gruss Lipper Biophotonics Center, Albert Einstein College of Medicine, Bronx, NY

Invadopodia, actin-polymerization driven protrusions of invasive carcinoma cells that focally secrete ECM-degrading proteases, are essential for tumor cell migration and intravasation in vivo during tumor cell dissemination from the primary tumor. Invadopodium formation involves the recruitment of a core complex of proteins including cortactin, cofilin, actin, N-WASP, and Tks5/FISH at the cell membrane. We have shown that cortactin phosphorylation at tyrosine residues, especially Y421, regulates the stability of this complex and promotes actin polymerization at invadopodium precursors. However, the mechanism by which cells regulate the cortactin phosphorylation-dephosphorylation cycle at invadopodia is unknown. Here we show that the invasion-specific Mena isoform, Mena^{INV}, is associated with a greater than 50 percent increase in phosphorylation of Y421-cortactin at invadopodia in human breast cancer cell lines after epidermal growth factor (EGF) stimulation. Little is known about the recruitment and localization of Mena isoforms to invadopodia, and we have found that Mena localizes to invadopodia before they begin degrading extracellular matrix (ECM). We show that Mena co-localizes at invadopodia with Riam, a scaffolding protein known to interact with Ena/VASP proteins similar to Mena, in a ring-distribution around invadopodia. We used acceptor-photobleaching FRET to show that Mena and Riam are in contact both at invadopodia and focal adhesions. We show that Mena initially localizes to the invadopodium core and later re-distributes to form a ring around the developing core complex, and that this pattern of localization is dependent on Riam and its closely-related family member Lamellipodin. Thus, Lamellipodin and Riam are involved in the recruitment of Mena to invadopodia, where Mena^{INV} specifically promotes invadopodium maturation by attenuating the dephosphorylation of cortactin at Y421. These results may explain the requirement for Mena^{INV} in invadopodium-dependent transendothelial migration during intravasation.

P1474**Board Number: B923****RhoG Negatively Regulates Invadopodia Formation.**S.M. Goicoechea¹, S. Awadia¹, A. Zynn¹, R. Garcia-Mata¹;¹Department of Biological Sciences, University of Toledo, Toledo, OH

Metastatic cells escape the tumor and enter the bloodstream by developing actin-rich membrane protrusions called invadopodia that degrade the extracellular matrix (ECM) to allow invasion of surrounding tissues. Even though many of the molecular components required for invadopodia formation have been identified, the signaling pathways that regulate these events are still poorly

understood. Invadopodia formation is controlled by the integrated activity of several members of the Rho family of GTPases, a family of proteins that regulates the actin cytoskeleton, but the molecular mechanisms that control their assembly are not known. Here we show that RhoG, a Rho GTPase related to Rac, functions as a negative regulator of invadopodia formation and functions during their disassembly, in a process that involves the exchange factor SGEF, paxillin and yet to be identified Rho-GAPs and effectors.

Our results show that silencing RhoG expression significantly increases the number of cells that form invadopodia in multiple breast cancer cell lines. In contrast, overexpression of RhoG inhibited the formation of invadopodia. Supporting these results, RhoG activity is significantly decreased during the formation of invadopodia. Silencing the expression of SGEF, a RhoG specific GEF, also increased the numbers of cells that formed invadopodia in a similar fashion than silencing RhoG. However, KD of other RhoG-GEFs, such as Ephexin 4 and PLEKHG6 did not affect the levels of invadopodia. These results suggest that SGEF functions to activate the pool of RhoG that negatively regulates the formation of invadopodia and that, for invadopodia to form, SGEF activity should be negatively regulated.

The molecular mechanisms by which RhoG regulates invadopodia formation are still under investigation, but we believe that paxillin phosphorylation is playing a role. Paxillin is a key component of invadopodia, and its phosphorylation plays a role in the disassembly of invadopodia. In RhoG KD cells, paxillin phosphorylation levels are significantly decreased, which suggests that the disassembly of invadopodia may be impaired in RhoG KD cells and would explain the increase in invadopodia number observed.

P1475

Board Number: B924

Microtubule acetylation regulates dynamics of KIF1C-powered vesicles and contact of microtubule plus ends with podosomes.

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Microtubule dynamics are important for a variety of key cellular functions such as intracellular trafficking, adjustment of the cell surface proteome, or adhesion structure turnover. In the current study, we investigate the effects of altered microtubule acetylation levels on the subcellular distribution of kinesins and actin cytoskeletal architecture in primary human macrophages. Microtubule acetylation was altered by overexpression or siRNA-induced depletion of the acetylase MEC-17, or by blocking alpha-tubulin deacetylation by addition of the inhibitor tubacin. We show that microtubule acetylation influences the subcellular distribution of vesicles associated with the kinesin KIF1C, as well as their directionality, velocity and run length. Moreover, tubulin acetylation alters the targeting frequency of microtubule plus ends on podosomes and influences the number of podosomes per cell and thus the matrix-degrading capacity of macrophages. Collectively, our results point to alpha-tubulin acetylation as

an important modification that impacts on kinesin vesicle dynamics, actin cytoskeletal architecture and cellular function of macrophages.

P1476

Board Number: B925

The heterodimer Kif3AC is a microtubule motor for polarized integrin trafficking and focal adhesion formation in migrating cells.

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In migrating cells, microtubules (MTs) contribute to front-back polarity that is essential for directional migration of cells in a variety of environments. MTs are thought to provide the tracks for directional delivery of membrane precursors and actin regulators necessary for protrusion of the leading edge. MTs also regulate the turnover of focal adhesions (FAs) by stimulating integrin endocytosis and FA disassembly through an endocytic process. However, some key questions remain to be addressed: How is FA disassembly coupled with reassembly? Is there a particular cytoskeletal mechanism to ensure that there is a polarized trafficking of internalized integrins during cell migration? Are there specific MT motors involved in this trafficking? Here we developed a system to study FA turnover and integrin recycling based on the synchronous endocytosis of integrins during FA disassembly triggered by MT regrowth. Endocytosed integrin recycled back to the cell surface and FAs reformed 30 min after FA disassembly. Strikingly, FA reassembly occurred in a very biased fashion towards the cell periphery/leading edge, suggesting polarized endocytic trafficking underlies FA reassembly. Following FA disassembly, integrins localized to Rab5 endosomes and to the Rab11-endocytic recycling compartment. Rab11 knockdown blocked integrin return to the cell surface and FA reassembly, establishing that FA reassembly requires integrin recycling in this system. The Rab11-FIP5 (Rab11 family of interacting proteins) and kinesin II have been previously shown to be involved in endocytic recycling so we tested if the members of the kinesin II family were involved in the polarized trafficking of integrins from the recycling compartment. We found that Kif3A or Kif3C, but not Kif3B depletion blocked FA reassembly without interfering with FA disassembly. Consistently, microinjection of Kif3A-tail blocked FA reassembly. Moreover, Kif3A-depleted cells displayed smaller and fewer FAs and reduced velocity in directed migration assays. In agreement with the hypothesis that a microtubule-associated motor drives integrin recycling, Kif3A localized to the Rab11-endocytic recycling compartment and Kif3A knockdown blocked integrin return to the cell surface after FA disassembly. Our study establishes for the first time a direct link between FA disassembly and reassembly through an endocytic recycling pathway. Moreover, our results support a mechanism in which both integrin endocytosis and recycling occur toward the cell front, thus maintaining a spatially polarized distribution of cycling integrins. To the best of our knowledge, Kif3AC is the first kinesin to be implicated in the polarized transport of integrins to the leading edge of migrating cells.

Ubiquitin and Proteasome Function

P1477

Board Number: B927

p53 Protects Against MG132-Induced Apoptosis in a Cell State-Dependent Manner.

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Since the approval of proteasome inhibitor, Bortezomib, for use in multiple myeloma in 2003, pharmacological proteasome inhibitors has been shown to be an important strategy for treating many types of cancer. While proteasome inhibitors induce apoptosis in certain rapidly proliferating cancer cells, some cells enters quiescence and survives. The mechanism by which quiescent cells become resistant to proteasome inhibitors is largely unknown. The tumor suppressor protein p53 plays an essential role in establishing and maintain quiescence in human cells. It also regulates several cell survival mechanisms, including cell cycle arrest, autophagy, and activation of reactive oxygen detoxifying enzymes. Here, we investigated the role of p53 in establishing resistance against proteasome inhibitors in quiescent cells. We found that MG132 (a know proteasome inhibitor) treatment induces the accumulation of p53 and its downstream target p21 more in quiescent cells than in proliferating primary human fibroblasts. shRNA mediated down-regulation of p53 sensitizes both proliferating and quiescent fibroblasts to MG132-mediated apoptosis and cell death, suggesting that p53 plays a protective role in all cell states. Additional experiments demonstrated that proliferating fibroblasts are susceptible to inhibition of cytoplasmic p53 activity, whereas quiescent fibroblasts are sensitive to inhibition of nuclear p53 activity. These findings suggest that p53 induces different protective mechanisms against MG132-mediated cell death, depending of the proliferative state of the cells. Autophagy, an alternative degradation pathway regulated by p53, is also induced in both cell states upon MG132 treatment. Identification of p53-mediated regulatory mechanisms that are activated in quiescent cells in response to proteasome inhibition may help to suggest strategies for improving the effectiveness of proteasome inhibitors in treating cancer.

P1478

Board Number: B928

Mule Ubiquitin Ligase Activity and the Tumor Suppressor ARF in Response to TNF.

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Being a key mediator of inflammation, TNF is involved in many pulmonary diseases, including asthma, COPD, ALI, and ARDS. TNF-induced JNK activation, a MAPK pathway that most notably leads to apoptosis

and inflammation, is shown to be specifically regulated by the E3 ubiquitin ligase Mule, and its polyubiquitinated target Miz1. Miz1 is a well-studied transcriptional factor; however, its novel cytoplasmic function acts as a signal and pathway-spec modulator or regulator that selectively suppresses JNK activation. Its E3 ubiquitin ligase Mule is involved in various processes including transcriptional regulation, apoptosis, DNA replication, and DNA damage response and repair. Mule leads to Miz1 proteasomal degradation by catalyzing the K48-linked polyubiquitination of Miz1. Interestingly, tumor suppressor ARF has been described to directly interact and strongly inhibits Mule ubiquitin ligase activity. ARF is a critical component of tumor surveillance; it functions as a checkpoint to prevent unrestricted cellular proliferation in response to aberrant oncogenic signaling. The mechanism behind ARF-Mule regulation is not yet known, and no prior studies have indicated whether this regulation can be controlled by extracellular stimuli. Our result shows that ARF inhibits Mule E3 ligase activity in response to TNF stimulation, indicating that the interaction between ARF and Mule is altered in response to TNF. We further confirmed the ARF-Mule interaction weakens upon TNF stimulation and that the polyubiquitination status of Miz1 coordinates with the release of ARF. To examine the possible modification that may affect Mule E3 ligase activity, we detected Mule tyrosine phosphorylation in response to TNF; and this event is required for ARF-Mule dissociation and Mule ubiquitin ligase activity. Furthermore, evidence suggests an emerging protein kinase plays a role upstream of ARF and Mule. Here, we report that the ARF-Mule interaction is regulated upon TNF stimulation via posttranslational modification of Mule, thus contributing to Mule ubiquitin ligase activity in Miz1 polyubiquitination and JNK activation.

P1479

Board Number: B929

Ubiquitin-Specific Protease 5 is necessary for *Drosophila melanogaster* development.

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Post-translational modification of proteins by the covalent attachment of ubiquitin (ubiquitination) plays an important role in numerous cellular pathways by altering protein function, location and stability. Ubiquitination is regulated by the actions of proteases known as deubiquitinating enzymes (DUBs). DUBs reverse protein ubiquitination and in turn regulate processes ranging from gene transcription to protein degradation. Ubiquitin-Specific Protease 5 (USP5) is a member of the ubiquitin-specific protease subclass of DUBs. The reported function of this protease is to hydrolyze unanchored (free) poly-ubiquitin chains and thereby recycle mono-ubiquitin for re-utilization. However, the physiological importance of USP5 *in vivo* is largely unclear. *Drosophila* USP5 is well conserved with its human counterpart. We show that, similar to its human orthologue, the fly USP5 hydrolyzes various types of ubiquitin-ubiquitin linkages rapidly *in vitro*. *In vivo* studies show that this DUB is developmentally required in the fruit fly: knockdown of USP5 throughout the fruit fly results in death during larval stages and this DUB is also physiologically important in glial and muscle cells, as well as in the fat body. At the biochemical level,

knockdown of USP5 causes an increase in poly-ubiquitin species without the depletion of mono-ubiquitin. In fact, expression of exogenous mono-ubiquitin does not suppress lethality caused by depleted USP5. Based on further data from genetic and biochemical assays, USP5 is required throughout fly development and may play a particularly important role in regulating general protein homeostasis.

P1480

Board Number: B930

The F-box protein FBXO25 plays a role in negative regulating of ELK-1 pathway in response to mitogens.

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FBXO25 is one of the 69 known human F-box proteins that serve as specificity factors for a family of ubiquitin ligases composed of SKP1, Rbx1, Cullin1, and F-box protein (SCF1) that are involved in targeting proteins for degradation across the ubiquitin proteasome system. Recently, we applied an in chip ubiquitination screen using a human protein microarray to uncover putative substrates for the FBXO25 protein and we identified, the c-fos protooncogene regulator ELK-1 as endogenous substrate for SCF1(FBXO25) E3 ligase. Also by phospho-kinase array approach, we found that overexpression in HEK293 cells of FBXO25 suppressed ERK1/2 phosphorylation in response to PMA. These results show that FBXO25 plays a combined effect in inhibition of MEK-ERK1/2-ELK-1 activation in response to mitogens. Further work involves screening of kinase-phosphatase RNAi library to identify FBXO25 target proteins implicated in the hypophosphorylation of ERK1/2.

P1481

Board Number: B931

The regulation of mitochondrial homeostasis by MARCH5-mediated ubiquitination on acetylated Mfn1.

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Mitochondrial dynamics and quality control have a central role in the maintenance of cellular integrity. Mitochondrial ubiquitin ligase membrane-associated RING-CH (MARCH5) regulates mitochondrial dynamics. Here, we show that mitochondrial adaptation to stress is driven by MARCH5-dependent quality control on acetylated Mfn1. Under mitochondrial stress conditions, levels of Mfn1 were elevated twofold and depletion of Mfn1 sensitized these cells to apoptotic death. Interestingly, overexpression of Mfn1 also promoted cell death in these cells, indicating that a fine tuning of Mfn1 levels is necessary for cell survival. MARCH5 binds Mfn1 and the MARCH5-dependent Mfn1 ubiquitylation was significantly elevated under mitochondrial stress conditions along with an increase in acetylated Mfn1. The

acetylation-deficient K491R mutant of Mfn1 showed weak interaction with MARCH5 as well as reduced ubiquitylation. Neither was observed in the acetylation mimetic K491Q mutant. In addition, MARCH5-knockout mouse embryonic fibroblast and MARCH5H43W-expressing HeLa cells lacking ubiquitin ligase activity experienced rapid cell death upon mitochondrial stress. Taken together, a fine balance of Mfn1 levels is maintained by MARCH5-mediated quality control on acetylated Mfn1, which is crucial for cell survival under mitochondria stress conditions.

P1482

Board Number: B932

Quantitative analysis of CRL function by single-molecule TIRF microscopy.

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Ubiquitin is a universal post-translational regulator of virtually all aspects of cellular function. Originally identified as a key part of the eukaryotic proteolytic system, ubiquitin-transfer to proteins is not inevitably a “kiss-of-death” signal but alternatively serves as a cue for ubiquitin-binding proteins (UBPs) that are integrated in numerous protein networks leading to complex subcellular rearrangements and differential signaling outputs. Ubiquitin conjugation to its target proteins involves the enzymatic activity of a myriad of E3 Ligase complexes. The most abundant E3 superfamily comprises the multi-subunit Cullin-RING ligases (CRLs). Unambiguous pairing of CRLs to their respective substrates has proven the key – and often rate limiting – step to fully understand the cellular role of these modular protein complexes. Technical difficulties to achieve this goal include short substrate residence times, rapid turnover of ubiquitinated targets and frequently low cellular abundance. To overcome these limitations we are pursuing a novel experimental route and adapt quantitative single molecule TIRF microscopy imaging to study CRL-substrate relationships. In particular, we aim to investigate novel CRL3 and CRL4 complexes required for cell cycle regulation, and exploit ultrasensitive detection of frequently transient and rare physiological E3 ligase–substrate encounters from cell extracts. A list of novel putative mitotic Cul4 substrate specific adaptors that emerged from in silico predictions and high-content RNAi screening previously conducted in our lab provides the biological foundation for this study. Plus, this single molecule imaging technique is accessible to recapitulate and quantitatively analyze general mechanistic aspects of dynamic E3 ligase function.

P1483**Board Number: B933****Arginyltransferase is critical for stress response and apoptosis and involved in cancer progression.**A. Kumar¹, M. Birnbaum¹, D.M. Patel¹, F. Zhu², Y. Guan², F. Zhang^{1,3};¹Molecular and Cellular Pharmacology, Univ Miami, Miami, FL, ²Department of Computational Medicine and Bioinformatics, Univ of Michigan, Ann Arbor, MI, ³Sylvester Comprehensive Cancer Center, Miami, FL

Arginyltransferase1 (Ate1) is an evolutionarily conserved enzyme that mediates posttranslational arginylation, which is the addition of an extra arginine to an existing peptide chain. The arginylation reaction changes the de facto primary sequence and surface charge of the target protein. It is known to lead to either ubiquitination and degradation, or functional modulation of the target. The in vivo activity of arginylation often increases during stressing conditions, such as heat shock, oxidative stress, or injury, all of which are known risk factors for cancers. However, as a posttranslational modification, arginylation is understudied and poorly understood. Currently, almost nothing is known about the role of arginylation or Ate1 in cancer or any other disease. Using multiple types of eukaryotic cells as test models, we found that knockout of Ate1 in cells leads to significantly reduced levels of apoptosis in the presence of stressing conditions. Conversely, over-expression of Ate1 leads to cell death. Therefore, our data indicate that arginylation is a pro-apoptotic factor. Intriguingly, we also found that the deletion of Ate1 gene in mammalian cells lead to a loss of contact inhibition and other cancer-like behaviors. Furthermore, a down-regulation of Ate1 leads to increased tolerance of reactive oxygen species (ROS) in prostate cancer cells, and that a lower level of Ate1 is linked to the metastasis of prostate cancer, where oxidative stress plays a major role in disease progression. Our data therefore uncover the novel role of Ate1 and arginylation as an regulator for stress response and apoptosis, with a potential involvement in the development of prostate cancers and other cancers.

P1484**Board Number: B934****Essential role of RecQL4 in checkpoint response to DNA damages in human cells.**S. Park¹, J. Im¹, Y. An¹, H. Choi¹, M. Hong¹, J. Lee¹;¹Dept of Biology Education, Seoul National University, Seoul, Korea

RecQL4 is a member of RecQ family helicase that plays important roles in maintaining genome integrity. Mutations in this gene are associated with Rothmund-Thomson Syndrome (RTS), which leads to developmental abnormalities, signs of premature aging, and high incidences of osteosarcoma. RecQL4 was shown to play multiple roles including the cellular response to DNA damage or oxidative stress, telomere maintenance, and DNA replication. RecQL4 comprises several domains. Distinct N-terminus of

RecQL4 showing limited homology to yeast Sld2 is essential for the initiation of DNA replication. While the function of helicase domain located in central part of RecQL4 is not well known, it appears to play important role in genome maintenance because most alternations of RecQL4 found in RTS patients are nonsense or frameshift mutations resulting in a truncated helicase domain. In this study, we examined the role of RecQL4 in checkpoint response to various DNA damages in human cells, and found that RecQL4 plays essential roles in checkpoint response. Essential roles of RecQL4 and its helicase activity in checkpoint responses will be discussed.

P1485

Board Number: B935

Unexpected Inherent Cellular Stress and Proteasome Sensitivity in Amyloidogenic Plasma Cells.

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Primary light chain amyloidosis (AL) is the most common form of systemic amyloidosis. AL is caused by a plasma cell (PC) clone producing unstable light chains (LC), which deposit in target tissues as amyloid fibrils and cause fatal organ failure. The leading therapies adopted against AL imitate the regimens for multiple myeloma (MM), the prototypical PC malignancy, including the first-in-class proteasome inhibitor (PI) bortezomib, aiming at reducing the PC clone, to limit toxic LC production (1). The recently established high clinical response of AL to PIs, nearing 90% complete remission when associated with alkylators, raises the attractive possibility that AL PCs are intrinsically vulnerable to drugs that perturb protein homeostasis. However, the relative unavailability of primary samples and suitable disease models hampers the investigation of the biology of AL PCs. We hypothesized that the unstable amyloidogenic LC could impair protein homeostasis and be stressful to PCs, sensitizing these cells to PIs. If correct, understanding the underlying molecular bases may disclose new therapeutic targets against AL. To test our hypothesis, we characterized, for the first time, patient-derived AL cells biochemically, functionally, and morphologically. Patient-derived AL PCs showed exquisite, in vitro unprecedented vulnerability to proteasome inhibition. As a result, they were significantly more sensitive to bortezomib-induced apoptosis than primary MM cells, paradigmatic PI-sensitive cancer cells. However, AL and MM cells showed comparable proteasome activity and proteosynthetic workload, two key determinants of PI sensitivity in PCs (2). To gauge the bases of PI sensitivity, we analyzed primary AL PCs by electron microscopy (EM) and EM cytochemistry, and disclosed that AL PCs display more abundant endoplasmic reticulum (ER) than primary MM cells, and have perinuclear mitochondria, revealing remarkable cellular stress. In view of the established role of autophagy, a lysosomal recycling cellular strategy, in protein and mitochondrial homeostasis (3), and of our recent discovery of adaptive ER-phagy in PCs (4), our evidence raises the possibility that AL LC production results in cellular stress by challenging autophagy.

To establish cause-effect relationships, we exploited an AL patient-derived cell line (ALMC1), and engineered a PC line to express patient-derived amyloidogenic and non-amyloidogenic LCs through a doxycycline-inducible system, to investigate the direct, cell-intrinsic effects of AL LC expression. Our models confirm the presence of higher cellular stress in amyloidogenic LC expressing cells, resulting in perturbed ER and mitochondria homeostasis. Ongoing functional characterization of AL cells holds promise towards the identification of novel molecular targets in AL.

1) Merlini et al., *Expert Rev Hematol.* 2014; 7(1):143-56. 2) Bianchi, Oliva et al., *Blood* 2009; 113(13): 3040-3049 3) Mizushima, Komatsu. *Cell* 2011; 147: 728–741 4) Pengo et al., *Nat Immunol.* 2013; 14(3): 298-305

P1486

Board Number: B936

Prolyl Hydroxylation and Glycosylation of the SCF-E3 Ubiquitin Ligase Complex Is Important for Protozoan Growth and Development at Physiological Oxygen Levels.

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Toxoplasma gondii is an obligate intracellular protozoan parasite that can proliferate in warm-blooded animal cells residing at 0.5-21% O₂, but the mechanism of adaptation is unknown. Previous studies showed that *Toxoplasma* depends on a cytoplasmic prolyl 4-hydroxylase (TgPhyA), which hydroxylates Skp1, an adaptor subunit of SCF (Skp1/Cullin-1/F-box protein)-class of E3 ubiquitin ligases, for optimal proliferation especially at low O₂. Disruption of Skp1 hydroxylation reduces its apparent Mr by approximately 1 kDa based on Western blotting which suggests hydroxylation-dependent additional modifications of Skp1 in wild-type parasites. We now provide tandem mass spectrometry evidence that TgSkp1 hydroxyproline is modified by a branched pentasaccharide. Contributions of two predicted cytoplasmic glycosyltransferases (TgGnt1 and TgPgtA) in TgSkp1 modification were confirmed by gel shift analysis and mass spectrometry of TgSkp1 from the disruption strains. Furthermore, the glycosyltransferase knockout strains exhibited reduced growth in fibroblast monolayers similar to those of TgPhyA-KO strains, suggesting that glycosylation may mediate the role of prolyl hydroxylation for optimal growth. Detection of substantial levels of the unmodified TgSkp1 peptide in Skp1 proteomic analyses of all strains implicate TgPhyA activity as rate limiting for TgSkp1 modification. Proteomic interactome studies revealed that the SCF complex exists in *Toxoplasma* and suggest that TgSkp1 hydroxylation and glycosylation regulates the abundance of assembled complexes. Finally, the Skp1 modification pathway is conserved with that of the social amoeba *Dictyostelium discoideum* but not in metazoans. Thus, as documented in *Dictyostelium*, hydroxylation-dependent glycosylation of TgSkp1 may be important for E3 SCF ubiquitin-ligase assembly and activity as the parasite senses changes in O₂-

availability. Moreover, our data indicate that TgPhyA O₂-sensing is a protist-specific mechanism suggesting that it may be a novel drug target.

Chaperones, Protein Folding, and Quality Control 2

P1487

Board Number: B937

Molecular mechanisms of hnRNPA1 and hnRNPA2 misfolding and toxicity.

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Missense mutations in heterogeneous ribonucleoproteins A1 and A2 (hnRNPA1-D262V and hnRNPA2-D290V) are strongly associated with inclusion body myopathy (IBM) with frontotemporal dementia (FTD), Paget's disease of bone (PDB), and amyotrophic lateral sclerosis (ALS)— a complex disorder known as multisystem proteinopathy (MSP). Thus, hnRNPA1 and hnRNPA2 join the ranks of RNA-binding proteins with “prion-like domains” (PrLDs) that are implicated in human neurodegenerative diseases. The mechanism by which they cause pathology in MSP is unknown. Both mutations occur within PrLDs and are predicted to enhance the prionogenicity of these proteins. In vitro experiments confirm that the wild-type proteins form self-seeding fibrils, and this process is accelerated in the mutated variants. I have established a yeast model that recapitulates the protein toxicity and mislocalization seen in disease. I have demonstrated that both hnRNPs require an intact RNA recognition motif (RRM) for toxicity, and that disruption of RNA-binding via missense mutations suppresses the toxicity of both proteins. The data suggest that, in the cytoplasm, both proteins must form aggregates to cause toxicity, but aggregate formation is not necessarily toxic.

P1488

Board Number: B938

Characterization of the Intracellular Behavior of COL4A5 and Clarification of Molecular Mechanism of Alport Syndrome.

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Alport syndrome (AS) is caused by mutation in COL4A3, COL4A4 or COL4A5, components of glomerular basement membrane (GBM). COL4A3/4/5 are secreted as heterotrimer. Restoring the normal COL4A3/4/5 network in the GBM is important for AS therapy; hence the need to investigate intracellular behavior of mutant COL4A5 such as degradation, heterotrimer formation and secretion. However, understanding COL4A3/4/5 regulation is poor due to a lack of cellular model for AS. Here, we establish AS cellular model and clarify the difference in degradation mechanism between wild type and mutant

COL4A5 proteins. We cloned and constructed human COL4A3/4/5 expression plasmids. These plasmids were transfected in HEK293 cells. We extracted whole cell lysates and checked the proteins' expression by western blotting. We made mutant COL4A5 plasmids by site-directed mutagenesis. We are now investigating whether wild type and mutant COL4A5 are degraded by proteasome or lysosome by treating transfected cells with inhibitors. COL4A5 protein stability is also checked by chase experiments. We constructed COL4A3/4/5 expression plasmids and detected these proteins' expression. Some mutant COL4A5 plasmids were successfully constructed. Now, we are investigating the difference in degradation mechanism between wild type and COL4A5 mutants. We made a novel AS cellular model, which expresses human COL4A3/4/5 proteins. This model is an important tool to investigate AS phenotype in molecular level such as protein stability, degradation mechanism, and heterotrimer formation of wild type or mutant COL4A5. Using this cellular model, we may be able to clarify the intracellular behavior of some mutant COL4A5, which could help to establish mutation-dependent personalized medicine.

P1489

Board Number: B939

The ribosome-associated complex antagonizes prion formation in yeast.

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The number of known fungal proteins capable of switching between alternative stable conformations is steadily increasing, suggesting that a prion-like mechanism may be broadly utilized as a means to propagate altered cellular states. To gain insight into the mechanisms by which cells regulate prion formation and toxicity we examined the role of the yeast ribosome-associated complex (RAC) in modulating both the formation of the *[PSI⁺]* prion – an alternative conformer of Sup35 protein – and the toxicity of aggregation-prone polypeptides. The Hsp40 RAC chaperone Zuo1 anchors the RAC to ribosomes and stimulates the ATPase activity of the Hsp70 chaperone Ssb. We found that cells lacking Zuo1 are sensitive to over-expression of some aggregation-prone proteins, including the Sup35 prion domain, suggesting that co-translational protein misfolding increases in $\Delta zuo1$ strains. Consistent with this finding, $\Delta zuo1$ cells exhibit higher frequencies of spontaneous and induced prion formation. Cells expressing mutant forms of Zuo1 lacking either a C-terminal charged region required for ribosome association, or the J-domain responsible for Ssb ATPase stimulation, exhibit similarly high frequencies of prion formation. Our findings are consistent with a role for the RAC in chaperoning nascent Sup35 to regulate folding of the N-terminal prion domain as it emerges from the ribosome.

P1490**Board Number: B940****Calreticulin gene expression is regulated by sumoylation in XBP-1-dependent mode in *Caenorhabditis elegans*.**Y. Lim¹, D. Lee², K. Kalichamy³, S. Hong¹, M. Michalak², J. Ahnn³, D. Kim¹, S. Lee⁴;¹Life Science, GIST, Gwangju, Korea, ²Biochemistry, University of Alberta, Edmonton, AB, ³Hanyang Univ, Seoul, Korea, ⁴BK21Plus BDR Team, Hanyang Univ, Seoul, Korea

Excessive accumulation of unfolded proteins in the endoplasmic reticulum (ER) lumen causes ER stress, which induces a set of genes, including those encoding ER-resident chaperones, to relieve the detrimental effects and recover homeostasis. Calreticulin is a chaperone that facilitates protein folding in the ER lumen, and its gene expression is induced by ER stress in *C. elegans*. Sumoylation conjugates small ubiquitin-like modifier (SUMO) proteins with target proteins to regulate a variety of biological processes, such as protein stability, nuclear transport, DNA binding, and gene expression. In this study, we showed that *C. elegans* X-box-binding protein 1 (Ce-XBP-1), an ER stress response transcription factor, interacts with the SUMO-conjugating enzyme UBC-9 and a SUMOylation target. Our results indicated that abolishing sumoylation enhanced calreticulin expression in an XBP-1-dependent manner, and the resulting increase in calreticulin counteracted ER stress. Finally, RNAi against *ubc-9* mainly affected the expression of genes associated with ER functions, such as lipid and organic acid metabolism. Our results suggest that sumoylation plays a regulatory role in ER function by controlling the expression of genes required for ER homeostasis in *C. elegans*.

P1491**Board Number: B941****The San1 and Ubr1 pathways in the quality control cytosolic protein folding.**R. Prasad¹, D.T. Ng^{2,3};¹Temasek Life Sciences Laboratory, Singapore, Singapore, ²Biological Sciences, National University of Singapore, Singapore, Singapore, ³Temasek Life Sciences Laboratory, Singapore, Singapore

Misfolded proteins in the cytosol are detected and degraded by the ubiquitin proteasome system through quality control pathways defined by specific E3 ubiquitin ligases. Although these pathways apparently work together as a comprehensive system to monitor the folding of most, if not all, cytosolic proteins, their respective contributions remain unknown. In budding yeast, two pathways have been established based around the E3 ligases San1 and Ubr1. San1 is a nuclear localized enzyme so its cytosolic substrates must traffic into the nucleus for ubiquitination and degradation. The localization of Ubr1 is unclear but some of its known folded substrates are nuclear suggesting that at least a fraction of Ubr1 functions in the nucleus. To bridge existing gaps and approach a unifying model of cytosolic protein quality control (CytoQC), the San1 and Ubr1 pathways were studied in detail. Interestingly, the chaperone requirements for both pathways are similar suggesting that the mechanisms upstream to

ubiquitination are the shared. These and new data on the relationship between the two pathways will be presented.

P1492

Board Number: B942

Role of Mouse Heat Shock Transcription Factor Isoforms in Zinc Ion Stress Pathways.

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The ability to handle low levels of environmental stressors is a key adaptive capacity of living things. Heat shock transcription factor 1 (HSF1) is the main regulator of stress pathways in response to heat, peroxide, and heavy metals such as cadmium and zinc ion. The goal of this project was to identify levels of zinc ions that would induce a stress response, then test whether Hsf1 isoform messenger RNAs vary in response to zinc treatment. Growth bioassays were carried out by treating NIH 3T3 cells in separate wells with different concentrations of zinc sulfate (0-640 μ M); surviving cells were identified using luminescence. Once appropriate treatment conditions were established, total RNA was isolated from treated and untreated cells, and complementary DNA was synthesized. To demonstrate that zinc induces the expression of heat shock protein (hsp) target genes, real-time RT-PCR (Taqman) assays were carried out. When cells are treated with 160 μ M zinc sulfate for 90 min, three hsp genes were activated (hsp70.1, 140-fold; hsp25, 8800-fold, and clusterin, 2-fold) compared to controls. To compare levels of expression of Hsf1 isoforms, we used specific primer sets to amplify the individual isoforms (Hsf1 alpha, beta, gamma, or delta) using PCR. The PCR products were analyzed by agarose gel electrophoresis. The levels of HSF1 alpha remain about the same in the presence and absence of zinc. Levels of Hsf1 delta and/or Hsf1 gamma may be down regulated by up to 50%. Levels of Hsf1 beta may be slightly upregulated in the presence of zinc. To further quantitate changes in Hsf1 isoform expression during zinc stress, we are developing isoform specific SYBR green assays. These experiments have established conditions for studying the role of heat shock factor 1 in metal stress response pathways in mouse cells. This work was supported by NIH NIGMS grant R15 GM096231 and Diversity Supplement.

P1493

Board Number: B943

Tagged HSF1 Gamma as a Tool for Tracking Heat Shock Components in Mouse Cells.

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Organisms must survive a variety of stressful conditions, including sudden temperature increases that damage important cellular structures and interfere with essential functions. Proteins activated in

response to elevated temperatures are called heat shock proteins (hsps). Heat shock proteins bind to misfolded proteins to repair them, promoting survival. The main regulator of genes for hsps is called Heat Shock Transcription Factor 1 (HSF1). Humans, mice, and other vertebrates express four different types of HSF1, called isoforms, which differ structurally in the presence or absence of either of two short regions of 28 and 22 amino acids. We have constructed sets of plasmids, which enable the individual mouse HSF1 isoforms to be expressed in tissue culture cells, with or without an activating deletion. Each set contains specific protein tags [either FLAG or HA] with one set tagged at the beginning (N-FLAG or N-HA) and another set tagged at the end (C-FLAG or C-HA) for each tag. The production of the tagged proteins was detected after transient transfection of NIH 3T3 cells via Western analysis. Three of the four sets are complete or mostly complete as confirmed by binding of HA or FLAG primary antibodies and later reprobing with HSF1 primary antibodies. The confirmed sets of plasmid constructs comprise highly specific tools for investigating HSF1 function in mouse cells. We are investigating the subcellular localization and hsp70.1 target gene activation of HSF1 isoform Gamma (δ) and Gamma Delta ($\delta\Delta$) using N-FLAG, C-HA, and C-terminal green fluorescent protein tagged versions. Based on immunofluorescence, in the absence of heat shock, CHA-tagged HSF δ is mainly present in the nucleus of cells. In contrast, CHA-tagged HSF $\delta\Delta$ is majorly present in the cytoplasm. In the presence of heat shock, CHA-tagged HSF δ is distributed more in the cytoplasm of a cell than in the nucleus. CHA-tagged HSF $\delta\Delta$ is entirely located in the cytoplasm when heat shock is present. This work was supported by a student-initiated research grant from SUNY Oneonta to A. M. and NIH NIGMS grant R15 GM096231 to N. J. B.

P1494

Board Number: B944

Defining a metazoan protein disaggregase for ALS disease proteins.

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Amyotrophic lateral sclerosis (ALS, also called Lou Gehrig's disease) is a debilitating, fatal neurodegenerative disease that is characterized by a severe and selective devastation of upper and lower motor neurons, progressive muscle weakness, paralysis, and death. Recently, ALS-linked mutations have been uncovered in several RNA-binding proteins with prion-like domains. Ordinarily, these proteins are localized to the nucleus. However, in ALS, mutations in different domains of these proteins lead to mislocalization of the proteins to the cytoplasm and formation of cytoplasmic inclusions in degenerating motor neurons. In the current study, we discovered that a nuclear import factor can function as a molecular chaperone to prevent the aggregation of these proteins by recognizing signal sequences in these RNA-binding proteins. Our results indicate that this nuclear import factor effectively inhibits the aggregation of WT disease proteins in vitro. The effectiveness of this nuclear import factor against disease protein aggregation is modulated by its interaction with the signal sequences. We show in our study that its activity is slightly impaired in disease mutants that have mutation in the signal sequence and is severely impaired in disease mutants that lack the signal sequences. We also

demonstrated in this study that this nuclear import factor is effective against aggregates that have different morphologies. Moreover, this nuclear import factor is shown to be able to reverse the aggregation of a subset of ALS disease proteins. Our results show that it has the potential to be developed as a general protein disaggregase for proteins bearing a signal sequence.

P1495

Board Number: B945

Using Tetracycline-Repression to Determine the Role of the J-protein Swa2 in [URE3] Prion Propagation.

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Many neurodegenerative diseases, such as Alzheimer's and Parkinson's disease, are associated with an insoluble protein conformation called amyloid. Investigating prions in the baker's yeast *Saccharomyces cerevisiae* provides insight into amyloid biology. A specific set of chaperone proteins in yeast (Sis1, Ssa1, and Hsp104) has been shown to be essential for prion propagation. Further, some prions have additional chaperone requirements, indicating that the role of chaperone proteins in prion propagation is heterogeneous. Unpublished studies from our laboratory have demonstrated that the J-protein Swa2 is required for propagation of the prion [URE3] and gene dissection analysis has revealed the minimum domain requirements of Swa2 necessary for successful [URE3] propagation. However, whereas gene dissection analysis reveals requirements, reducing Swa2 expression *in vivo* should provide mechanistic information about the specific function of Swa2 in prion propagation. As expected, our initial repression experiments demonstrated that aggregates of [URE3] are cured upon addition of drug and subsequent decrease in Swa2 expression, further confirming that Swa2 protein expression is necessary for [URE3]. Surprisingly, these experiments also revealed curing in cultures without added doxycycline. Upon further investigation, it was determined that the tetracycline-repressible promoter significantly overexpresses Swa2, resulting in curing of [URE3] by generic J-protein overexpression. We subsequently optimized the experimental conditions and drug dosage to initiate experiments with Swa2 expression near wild-type levels. Using this optimized tetracycline-repression method which stabilizes [URE3], Swa2 expression can be fully repressed after approximately twelve generations upon supplementation with additional drug. While a colony color phenotype allows the measurement of curing kinetics, we are employing a Ure2-GFP chimera that will allow us to monitor changes in location and morphology of the biological aggregates *in vivo*. We are also optimizing a Semi Denaturing Detergent Agarose Gel Electrophoresis (SDD-AGE) protocol to monitor changes in amyloid polymer size and supplement the data gathered from GFP studies. We anticipate that this approach, in combination with homology studies, will expand upon our understanding of both molecular chaperone functions and amyloid biology.

P1496**Board Number: B946****Structural homology analyses of the tetratricopeptide repeat region of the yeast auxlin homolog Swa2 reveal considerable structural conservation with human HOP1.**E.E. Oliver¹, M.E. Rockman¹, J.K. Hines¹;¹Chemistry, Lafayette College, Easton, PA

Previous results from our laboratory assert that the yeast Hsp40 chaperone protein Swa2 is essential for the propagation of the prion [URE3] in a yeast population. Two regions of the protein are both necessary and sufficient to maintain [URE3] in these populations: the J-domain and the tetratricopeptide repeat (TPR) region. The J-domain is characteristic of all Hsp40 proteins and is known to interact in a complex with Hsp70, strongly suggesting that Swa2 acts in conjunction with Hsp70 in [URE3] propagation. However, the function of the TPR domain in Swa2 is undetermined. TPR domains typically act as docking platforms for a variety of protein complexes. As such we propose that the Swa2 TPR domain either participates in a bipartite interaction with Hsp70, analogous to the C-terminal domains of other yeast Hsp40s, or that the TPR domain mediates the formation of a ternary complex with another undetermined protein in [URE3] propagation. Pair-wise sequence alignments of the Swa2 TPR region to the TPR1 and TPR2A domains of the human HOP1 protein, which interact with human Hsp70 and Hsp90 proteins respectively, showed significant residue identity indicating a conservation of overall tertiary structure between all three domains. Although the Swa2 TPR region is more similar to TPR1 than TPR2A, residues which are likely to mediate protein-protein interactions had yet to be identified and characterized. To do this, we analyzed the crystal structures of the TPR1 and TPR2A domains co-crystallized with peptide ligands in conjunction with our sequence alignments. Our analyses uncovered a high degree of sequence similarities in key charged residues that likely form the two-carboxylate clamp, strongly suggesting that Swa2's TPR region will bind the canonical EEVD motif found on in yeast Hsp70 and Hsp90, and many other proteins. The analyses also suggested that Swa2 may specifically recognize the C-terminal dicarboxylate of the C-terminal aspartate, indicating that Swa2's in vivo binding partner likely possess a C-terminal EEVD motif. The capacity to form hydrophobic interactions that determine specificity between Hsp70 and Hsp90 was clearly evident but no conclusion about specificity between these two proteins can be currently drawn. However, this analysis has identified candidate residues for mutagenesis to test potential interactions with yeast Hsp70 and Hsp90. We expect that understanding the function of the Swa2 TPR domain will provide new insight into both J-protein function and the complex mechanisms of prion propagation.

P1497**Board Number: B947****Yeast prions and lipid profiles: protein-only inheritance and control of membrane lipid composition.**Q.H. Bui¹, J. Sherma¹, B. Fried², J.K. Hines¹;¹Chemistry, Lafayette College, Easton, PA, ²Biology, Lafayette College, Easton, PA

A central characteristic of all prions is that they are self-templating: once formed, the prion aggregate recruits additional monomeric protein into the growing fiber. Yeast prions of the species *Saccharomyces cerevisiae* stand as an example of the inheritance of complex traits based solely on the acquisition of a cytoplasmic protein aggregate as they confer distinctive phenotypes to the cells which harbor them, creating heterogeneity within an otherwise clonal cell population. These phenotypes typically arise from a loss-of-function of the prion protein which is unable to perform its normal cellular function while sequestered in prion amyloid aggregates. For example, the prion [PSI⁺], formed by the protein Sup35, broadly affects the proteome of yeast cells that harbor it by causing widespread nonsense suppression due to the sequestration of Sup35, a ribosomal translation termination factor. The physiological consequences of prion formation are largely undetermined given the recent discovery of most yeast prions within only the past five years. To begin to address this issue, we initiated a direct investigation into the potential control that yeast prions exert over fungal lipid content. Following a total lipid extraction, we utilized silica gel HPTLC (high performance thin-layer chromatography) to conduct pairwise quantifications of the relative levels of free sterols, free fatty acids, steryl esters, squalene, phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine in otherwise clonal [PSI⁺]-infected and [PSI⁺]-cured ([psi⁻]) cells. Preliminary analyses with n≥4 replicates have revealed differences (p

P1498**Board Number: B948****A potential role of the 'Anti-prion J-protein 1' (Apj1) in the curing of the prion [PSI⁺] by Hsp104 overexpression.**M.T. Astor¹, Z.A. Sporn¹, J.K. Hines¹;¹Chemistry, Lafayette College, Easton, PA

The prions of *Saccharomyces cerevisiae* are heritable aggregates of misfolded yeast proteins. Yeast prions are incorporated into daughter cells through the fragmentation of these aggregates by molecular chaperone proteins, including J-proteins, Hsp70s, and Hsp104. Interestingly, previous studies have shown that the overexpression of Hsp104 cures only the prion [PSI⁺]. By studying the genetic interactions between J-proteins and Hsp104, we aim to better understand how the action of multiple chaperones results in the curing of only this specific and otherwise highly stable prion. In order to clarify the role of J-proteins, we asked whether any of the other 12 J-proteins located in the *S. cerevisiae*

cytosol are also necessary for Hsp104-induced [PSI⁺] curing. We also investigated whether prion amyloid structure (variant identity) affects experimental outcomes. In order to do this, we first demonstrated that either of two weak [PSI⁺] variants, Sc37 and VL, can be maintained in the absence of each J-protein other than Sis1 using mating experiments. Subsequently, curing of weak [PSI⁺] by Hsp104 overexpression was examined, and [PSI⁺] was cured in all strains, demonstrating that no J-protein other than Sis1 is necessary for the curing of weak [PSI⁺] by overexpression of Hsp104. This analysis was next repeated using a strong [PSI⁺] variant, STR. In this case, previous studies have already shown that deletion of these individual J-proteins does not affect [PSI⁺]STR propagation, allowing us to again examine curing by Hsp104 overexpression in each deletion strain. [PSI⁺]STR was cured upon Hsp104 overexpression in all J-protein deletion strains, however we noted exceptional resistance to Hsp104 mediated curing in the strain lacking Apj1 (originally named: Anti-Yeast Prion J-protein 1) specifically. Apj1 has been implicated in prion biology several times; often acting similarly to Ydj1 in prion biology. Here we find a novel genetic interaction for Apj1 and prions that is distinct from Ydj1. Additional experiments at the level of domains will allow further characterization of a potential role for Apj1 in Hsp104-mediated [PSI⁺] curing and may help to explain the prion-specificity of this curing mechanism.

P1499

Board Number: B949

Domain and homology studies of the J-protein Sis1 in the curing of [PSI⁺] by overexpression of Hsp104.

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The purpose of this investigation was to examine the yeast cytoplasmic Hsp40 protein Sis1 and its homologs with regard to their role in the curing of the [PSI⁺] prion due to overexpression of Hsp104. Yeast prion propagation requires the chaperone proteins Sis1, Ssa1, and Hsp104. Interestingly, when Hsp104 is overexpressed the [PSI⁺] prion is lost, or “cured”, from the population in a poorly understood but Sis1-dependent manner. The mechanism of [PSI⁺] curing by Hsp104 overabundance is currently the subject of significant debate in literature. We recently reported that the human homolog of Sis1, Hdj1, was capable of substituting for Sis1 in the propagation of strong but not weak [PSI⁺] variants. Here, we have extended that investigation to explore the potential of both Hdj1 and the *Drosophila melanogaster* homolog Droj1 to substitute for Sis1 in the curing of [PSI⁺] by Hsp104 overexpression. In addition, we also considered the potential effects of yeast genetic background and controlled for polymorphisms by utilizing two distinct yeast strain backgrounds commonly used in yeast prion investigations. We found that like Hdj1, Droj1 only substitutes for Sis1 in the propagation of strong variants; two weak variants, Sc37 and VL, were lost when Droj1 was expressed as the sole Sis1 homolog whereas two strong variants, Sc4 and VH, were maintained without any distinct phenotype in both yeast genetic backgrounds tested. Upon transformation with a plasmid overexpressing Hsp104, we found that both strong variants were maintained, again in both backgrounds, indicating that Droj1 is incapable of substituting for Sis1 in Hsp104-mediated curing. Hdj1 behaved similarly to Droj1 except that, surprisingly, the variant Sc4 was

cured, but only in the 74D-694 background, indicating that while Hdj1 is clearly deficient in replacing Sis1 in this mechanism, the combination of a specific yeast genetic background and prion variant alters the experimental outcome. Notably, the finding that the same variant was stable when Droj1 was present instead of Hdj1 indicates that Hdj1 has a gain of function in that genetic background, rather than an alternative model in which the curing mechanism simply becomes Hsp40-independent. Finally, because we found differences due to both yeast genetic background and prion variant identity, we also conducted an additional investigation of the Sis1-domain requirements for Hsp104-mediated [PSI⁺] curing, expanding upon another prior investigation by extending the analysis to multiple [PSI⁺] variants and genetic backgrounds. In accordance with our most recent published investigation, we found drastic differences in the requirement for Sis1 in Hsp104-mediated [PSI⁺] curing between weak and strong [PSI⁺] variants.

P1500

Board Number: B1000

Glycobiological aspects of cellular stress responses.

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Mammalian cells adaptively respond to exogenous stressors through a number of conservative signaling pathways that activate specific transcription factors leading to the upregulation of genes required for either cell survival or programmed cell death. A novel conceptual paradigm has recently emerged suggesting the contribution of glycocalyx remodeling to maintain cell homeostasis. It was hypothesized that stress-dependent changes in the expression of cell surface glycans to endogenous lectins (glycan-binding proteins) represents a distinctive level of glycobiological regulation of cellular functions. To test this hypothesis, we used a human promyelocytic leukemia cell line HL-60 as a model and examined the effects of three different stress stimuli, mimicking oxidative stress (0.01 mM menadione), hypoxia (0.1 mM cobalt chloride), and endoplasmic reticulum stress (0.001 mg/mL tunicamycin), on the cell adhesion to immobilized plant lectins with different glycan-binding specificities, as well as examining the expression of eleven galectin genes (LGALS 1, 2, 3, 4, 7, 8, 9, 10, 12, 13, and 14). Suspension culture of HL-60 cells was maintained in Iscove's modified DMEM supplemented with 10% FBS and then exposed for 24h to the stress drugs, rinsed with PBS, and used either directly for cell adhesion assay or for total RNA extraction, cDNA synthesis, and conventional PCR. Glycoprofiling of HL-60 cells revealed a strong adhesion of untreated cells to immobilized lectins from *Canavalia ensiformis*, *Sambucus nigra*, and *Ricinus communis* while the adhesion to fibronectin and lectins from *Arachis hypogaea*, *Dolichos biflorus*, *Glycine max*, *Triticum vulgare*, and *Ulex europaeus* was relatively weak. All three stress treatments were found to increase significantly the adhesion to *Triticum vulgare* agglutinin; in addition menadione significantly increased adhesion to *Sambucus nigra* agglutinin. Stress-induced changes in the expression of galectin genes were more diverse allowing us to classify them tentatively in three categories: upregulated (3, 10, and 12), downregulated (1 and 9), and constitutive (stable expression of 8 and 14 as well as not detectable 2, 4, 7, and 13) galectins. Although this classification of galectins was

not uniform between different stress treatments, it provides a new starting point to recognize the complexity of galectin networks in cells. As such, it is not surprising that our preliminary bioinformatics analysis revealed non-overlapping diversity in putative transcription factors and miRNAs regulating the expression of galectins. Collectively, our results provide a strong support to the conceptual idea that differential expression of specific glycans and galectins can serve as a molecular signature of cellular stress responses.

P1501

Board Number: B1001

Oncogene de-addiction from Hsp90 induces senescence-like phenotype in malignant and metastatic tumor cells.

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Bypassing senescence in normal cells is being achieved by activation of multiple signal transduction pathways. We studied how Hsp90 favours oncogene addiction using primary, malignant and metastatic tumor cells. Between the wild type and mutant Raf, mutant Raf induced senescence in primary cells and malignant tumor cells, but enhanced proliferation in metastatic cells. However, inhibition of Hsp90 induced cell death in metastatic cells suggesting that oncogene addiction requires Hsp90. With increased Raf expression, we observed an increase in active Hsp90. Examining the interaction between different forms of Raf with deletion constructs of Hsp90 revealed that the highly charged middle region of Hsp90a, called the “hinge region” is responsible for the Raf interaction and stabilization. Interestingly, the constitutive form of Hsp90, the Hsp90b is found to play no role in the oncogene addiction. Further, Hsp90 has more affinity towards the mutant Raf over the wild type Raf. In contrast to the previous reports that Hsp90 interacts with the kinase clients through a kinase-kinase interaction domain, for the first time we demonstrate that the Hsp90 kinase interacting domain is indispensable for Raf interaction. Promoting “proliferative crisis” by overexpressing mutant forms of Raf in tumor cells had survival advantage, due to active Hsp90. However, Raf expression in primary cells lacking active Hsp90 resulted in senescence. We also demonstrated that Raf induced senescence requires intact DNA damage response (DDR) and functional p53. Pre-sensitizing these cells to Hsp90 inhibition enhanced senescence. Our findings indicate adaptive roles of Hsp90 to oncogene addiction, and a new perspective of using Hsp90 inhibitors to promote senescence.

P1502**Board Number: B1002****Sigma-1 Receptor Deficiency Hinders p35 Degradation and Causes Impaired Neuronal Circuitry.**S.A. Tsai¹, M.J. Pokrass¹, T. Su¹;¹NIDA, NIH, Baltimore, MD

Cyclin-dependent kinase (cdk5) is a serine/threonine directed kinase that performs signaling roles in neuronal survival, migration, synaptogenesis, and axon guidance. Cdk5 activity is dependent on its activator, p35, which is a short-lived ($t_{1/2} = 20$ min.) neuronal-specific peptide that is anchored to the plasma membrane (PM) via an N-terminal myristoylation motif. Dysregulation of cdk5 has long been implicated in the pathophysiology of neurodegenerative disease because it causes abnormal hyperphosphorylation of cytoskeletal proteins such as tau and neurofilament, which ultimately leads to the emergence of neurofibrillary tangles. Thus, p35 is a tightly regulated protein that undergoes rapid proteasomal degradation; however, neurotoxic insults promote p35 conversion to p25. p25 also activates cdk5, though it possesses a longer half-life and is no longer anchored to the PM, allowing atypical phosphorylation by cdk5. The sigma-1 receptor (Sig-1R) is an endoplasmic reticulum (ER) molecular chaperone that is implicated in several diseases of the central nervous system such as Alzheimer's disease, drug addiction, and depression. Neuroimaging studies have revealed that brains of Alzheimer's and Parkinson's patients are deficient in Sig-1R expression; however, the specific role of the Sig-1R in neurodegeneration is still unclear. This study examined the role of Sig-1Rs in the regulation of p35. We found that Sig-1R siRNA (siSig-1R) treated neurons exhibit greater basal levels of p35 expression and that this increase is likely due to a decreased degradation rate of p35. siSig-1R neurons also display shorter axonal length and disoriented axonal projection. Treatment with myristate restored axonal elongation in the Sig-1R KO neurons, although myristate itself may promote axonal growth. Furthermore, we show that myristic acid competes with the Sig-1R agonist (+)-pentazocine for Sig-1R binding. Taken together, these findings indicate that Sig-1Rs mediate the myristoylation of p35, thereby regulating the degradation of p35, which, in turn modulates cdk5 activity. This property of the Sig-1R appears important to the structural and functional maintenance of the axon.

P1503**Board Number: B1003****Subcellular fractionation analysis of the extraction of ubiquitinated polytopic substrates during ER-associated degradation.**K. Nakatsukasa¹, T. Kamura¹;¹Division of Biological Science, Nagoya University, Nagoya, Japan

During ER-associated degradation (ERAD), misfolded polytopic membrane proteins are ubiquitinated and retrotranslocated to the cytosol for proteasomal degradation. The mechanisms underlying the

recognition and ubiquitination of polytopic substrates have been well-documented; however, the events associated with the post-ubiquitination step of polytopic substrates remain largely unknown. To better define the localization and physical properties of ubiquitinated polytopic substrates during ERAD, we performed subcellular fractionation analyses of ubiquitinated Ste6p (star), a 12 transmembrane domain-containing ERAD substrate. Consistent with previous studies, the extraction of ubiquitinated Ste6p* from the ER membrane was dependent on Cdc48p/p97. Ubx2p, which recruits Cdc48p/p97 to the ER, facilitated the extraction of Ste6p (star). By contrast, the downstream components of Cdc48p/p97, including Rad23p/Dsk2p, and the proteolytic activity of the proteasome were dispensable for the extraction. Lipid droplet formation was not required for the degradation of Ste6p (star). Sedimentation and flotation assays showed that ubiquitinated Ste6p* in the cytosol was free from membranes and most likely resides in a protein complex. Our results suggest that the solubility of polytopic substrates in the cytosol is maintained by proteinous factors. The fractionation assays developed in the present study provide a means to further dissect the ill-defined post-ubiquitination step during ERAD of polytopic substrates.

P1504

Board Number: B1004

Identification of three different structures of human alpha synuclein relevant to Parkinson disease.

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Alpha synuclein (aSyn) is the major component of Lewy bodies and Lewy Neurites which are pathological markers for synucleinopathy including Parkinson's disease. The physiological structure of aSyn has been controversial from natively unfolded structure(s) to a stable helical tetrameric structure. It has been known that aSyn could also exist as membrane bound forms, dimer/oligomer, and amorphous/amyloid aggregates. Here we examined various structures of aSyn polypeptide formed under varied conditions. The resultant structures were analyzed mainly by deuterium hydrogen exchange mass spectrometry, which has been proved to be excellent in analyzing many metastable and amorphous protein structures. All deuterium-labeled aSyn was prepared by incubating aSyn in 100% deuterium oxide at 45°C for 30 min. The deuterated aSyn was incubated in H₂O buffer under the varied conditions, and their structures were evaluated by deuterium to hydrogen exchange mass spectrometer with highly efficient online protease HPLC system. More than 100 peptides covering whole 140 residues were successfully obtained. N-terminal and NAC domains of aSyn could form three different conformations with different exchange rate; low, medium, high protection values. The structure with low protection seems to be similar to (pre)molten globule-like structure. The structure with high protection resembles a highly organized structure like helical dimer or tetramer. On the other hand, the structure with medium stability may be metastable under the condition examined, because it is only partially detected between two other structures. For all three structures, C-terminal domain (~100~140) of aSyn shows little protection values

close to those of corresponding peptides, indicating that it exists as random disordered structure. It is suggested that aSyn could be converted via this metastable intermediate structure from unfolded to (pre)molten globule to highly organized structures.

P1505

Board Number: B1005

Inhibition of ER Ca²⁺ release or the p38 SAPK inhibits UPR signaling and apoptosis activation during moderate ER stress.

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When unfolded proteins accumulate in the endoplasmic reticulum (ER), stress signaling pathways collectively called the Unfolded Protein Response (UPR) are induced. Modeling of ER stress in cultured cells is often performed with high doses of poisons that arrest essential functions within the ER. In examining the potential implications of chronic ER stress signaling in organismal models, a modest ER stress would be anticipated as clinically appropriate. In this study, the ER was challenged with a 50-fold range of tunicamycin, an inhibitor of N-linked glycosylation. Tunicamycin induced cellular apoptosis and stress signaling over a range of concentrations from 20 nM to 1000 nM. Increasing doses of tunicamycin resulted in similar increases in activation of signaling from the ER-localized IRE1 transmembrane protein, expression of UPR transcriptional targets, and activation of the p38 stress activated protein kinase. High doses of tunicamycin were shown to cause an elevation in cytosolic Ca²⁺, while modest doses showed no significant changes in cytosolic Ca²⁺ levels. Interestingly, inhibition of Ca²⁺ release from the inositol trisphosphate receptor was found to inhibit ER stress signaling and apoptosis only at modest doses of ER stress (20-40 nM). During moderate ER stress, p38 inhibition was sufficient to inhibit UPR-based increases of GRP78 expression and the activation of cellular apoptosis. This work supports a model in which moderate ER stress uses Ca²⁺ release and p38 signaling as a central component of both UPR activation and a subsequent ER stress-induced apoptosis.

P1506

Board Number: B1006

Suramin Inhibits Hsp104 ATPase and Disaggregase Activity.

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Hsp104 is a hexameric AAA+ protein that utilizes energy from ATP hydrolysis to dissolve disordered protein aggregates as well as amyloid fibers. Interestingly, Hsp104 orthologues are found in all kingdoms of life except animals. Thus, Hsp104 could represent an interesting drug target. Specific inhibition of

Hsp104 activity might antagonize non-metazoan parasites that depend on a potent heat shock response, while producing little or no side effects to the host. However, no small molecule inhibitors of Hsp104 are known except guanidinium chloride. Here, we screen over 16,000 small molecules and identify 16 novel inhibitors of Hsp104 ATPase activity. Excluding compounds that inhibited Hsp104 activity by non-specific colloidal effects, we defined Suramin as an inhibitor of Hsp104 ATPase activity. Suramin is a polysulphonated naphthylurea and is used as an antiprotozoal drug for African Trypanosomiasis. Suramin also interfered with Hsp104 disaggregase, unfoldase, and translocase activities, and the inhibitory effect of Suramin was not rescued by Hsp70 and Hsp40. Suramin does not disrupt Hsp104 hexamers and does not effectively inhibit ClpB, the *E. coli* homolog of Hsp104, establishing yet another key difference between Hsp104 and ClpB behavior. Intriguingly, a potentiated Hsp104 variant, Hsp104A503V, is more sensitive to Suramin than wild-type Hsp104. By contrast, Hsp104 variants bearing inactivating sensor-1 mutations in nucleotide-binding domain (NBD) 1 or 2 are more resistant to Suramin. Thus, Suramin depends upon ATPase events at both NBDs to exert its maximal effect. Suramin could develop into an important mechanistic probe to study Hsp104 structure and function.

P1507

Board Number: B1007

The molecular chaperone Hsp70 binds to and stabilizes Nod2, an important protein involved in Crohn's disease: Identifying a small molecule stabilizer.

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Microbes are detected by the pathogen-associated molecular patterns (PAMPs) through specific host pattern recognition receptors (PRR). Nucleotide binding oligomerization domain-containing protein 2 (Nod2) is an intracellular PRR that recognizes fragments of bacterial cell wall. Nod2 is important to human biology, as when it is mutated it loses the ability to respond properly to bacterial cell wall fragments, as observed in Crohn's disease. In order to determine the mechanisms of misactivation in the Nod2 Crohn's mutants, we developed a cell based system to screen for protein-protein interactors of Nod2. We identified heat shock protein 70 (Hsp70) as a protein interactor of both wild-type and Crohn's mutant Nod2. Hsp70 has previously been linked with inflammation, especially in the regulation of anti-inflammatory molecules. Induced Hsp70 expression in cells increased Nod2 response to bacterial cell wall fragments. In addition, a Hsp70 inhibitor, KNK437, was capable of decreasing a Nod2 mediated NF- κ B activation in response to bacterial cell wall stimulation. We found Hsp70 to regulate Nod2's half-life, as increasing the Hsp70 level in cells increased Nod2's half-life, and down-regulating Hsp70 decreased Nod2's half-life. The expression level of the Crohn's associated Nod2 variants were less compared to wild-type. The over-expression of Hsp70 significantly increased Nod2 levels as well as the signaling capacity of the mutants. Thus our study shows that restoring the stability of the Nod2 Crohn's mutants is sufficient for rescuing the ability of these mutations to signal the presence of a bacterial cell wall ligand. Further, we have identified the minimal region of Hsp70 that facilitates the interaction with

Nod2. Studies are currently under way to identify a pharmacoperones that can potentially stabilize the Crohn's associated Nod2 variants.

Physical Approaches to Cell Biology

P1508

Board Number: B1009

Cytoplasmic freezing – a novel cytoplasmic state in fission yeast.

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The cytoplasm is generally seen as a passive, viscoelastic material that is enclosed by the cell membrane and with characteristics that are defined by the nature and the density of its content. A recent essay discussed evidence suggesting that the cytoplasm should not be seen as a simple fluid but rather as a poroelastic material with a fluid and a solid phase [1]. Similarly, it was hypothesized in another context that liquid phase separation may be a fundamental principle of cytoplasm organization [2]. Both publications imply the possibility that cells can actively regulate the physical properties of their cytoplasm, even locally, which would provide a novel way to contribute to mesoscale organization of the cell. Yet, good experimental evidence or even molecular mechanisms for such control of cytoplasmic states have not been found. Here we show that such active control of cytoplasm viscosity occurs. We present a novel state of cytoplasmic organization, in which the cytoplasm transitions from fluid-like to solid-like. We therefore term this phenomenon cytoplasmic freezing. The frozen state is characterized by the fact that the diffusive motion of observable structures in the cytoplasm is completely restricted and that optical tweezers can no longer move lipid granules. In addition, cell wall removal does not result in the loss of cylindrical cell shape as occurs in normal cells. The frozen state occurs in fission yeast cells in advanced glucose starvation and rapidly reverses upon glucose addition. The dramatic immobilization of all structures suggests the formation of a fine polymer network. The primary candidates for the formation of such a network would be proteins known to form filament networks like actin, microtubules or septins, but based on our single particle tracking, optical tweezers, fluorescence microscopy, and electron microscopy data, we discard all of them.

[1] Mitchison, T.J. et al., *Seminars in Cell & Developmental Biology* 19 (2008), 215–223

[2] Hyman, AA et al., *Developmental Cell*, 21 (2011), 14-16

P1509**Board Number: B1010****Computing cytoplasmic flows during pronuclear migration and spindle positioning in C-elegans embryo.**E. Nazockdast¹, D. Needleman², M. Shelley¹;¹Courant Institute, New York University, New York, NY, ²School of Engineering and Applied Sciences, Harvard University, Cambridge, MA

We investigate theoretically models of pronuclear migration and spindle positioning in single cell *Caenorhabditis elegans* embryos using new and highly efficient numerical techniques. These methods fully resolve the long-ranged hydrodynamic interactions, mediated by the cytoplasm, between microtubules and with other cytoskeletal objects, and account for microtubule flexibility and dynamic instability. To do this, many-body fluid-structure interactions are computed using a highly efficient and scalable fast multipole method for the Stokes equations of viscous flow, which allows simulating thousands of fibers. Different types of molecular motors can be incorporated into our framework. We explore pronuclear migration and spindle positioning in single cell *Caenorhabditis elegans* embryos using two previously proposed positioning mechanisms: cytoplasmic pulling upon MTs and MTs pushing upon cortex. While our simulations show proper positioning occurs in both models, their associated cytoplasmic flows are fundamentally different. This suggests that cytoplasmic flow visualization during pronuclear migration and spindle positioning could differentiate between different proposed mechanisms. We also investigate the consequences of varying catastrophe and rescue rates, microtubule rigidity, and the shape of embryo.

P1510**Board Number: B1011****The nucleus is an intracellular propagator of actomyosin forces in NIH 3T3 fibroblasts.**S. Alam¹, D. Lovett², K.J. Roux³, R.B. Dickinson², T.P. Lele²;¹Chemical Engineering, Univ Florida, Gainesville, FL, ²Chemical Engineering, University of Florida, Gainesville, FL, ³Children's Health Research Center, Sanford Research, Sioux Falls, SD

How the migrating cell positions its nucleus is not understood. Previous studies with engineered lamellipodia suggested that the nucleus is pulled forward by anterior actomyosin contraction in crawling fibroblasts. Here we show that the nucleus deforms due to the formation of local protrusions proximal to the nuclear surface. The deformation relaxed on collapse of the local protrusion, and required actomyosin activity and an intact LINC complex. Femtosecond laser ablation of lateral stress fibers that run parallel to the side of the nucleus and appear to be touching did not result in a lateral expansion of the nucleus, arguing against a mechanism in which the protrusions pull on compressive fibers and allow the nucleus to locally expand. Instead, we propose that actomyosin contraction in the protrusion pulls

on the nuclear surface to move it. Consistent with this mechanism, we found that the nuclear position closely coincided with the point of maximum tension in the cell (quantified from traction force microscopy), whereas it lagged behind it by several microns in LINC complex disrupted cells. To further challenge the hypothesis that the nucleus transmits tension from the anterior to the posterior of a migrating cell, we detached the trailing edge with a micropipette and quantified the drop in the traction stress in the anterior region of the cell. The drop in the traction stress at the front was significantly larger in normal cells as compared to LINC complex disrupted cells. Collectively, these results are consistent with a model in which nuclear position in migrating fibroblasts is determined by a tug-of-war between anterior and posterior actomyosin pulling forces.

P1511

Board Number: B1012

Super-resolution imaging reveals structural determinants underlying genetic polymorphisms that influence cellular plasticity and disease susceptibility.

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Visualization of specific molecules and their interactions in real time and space is essential to delineate how cellular dynamics and signaling circuit are orchestrated. Spatial regulation of conformational dynamics and structural plasticity of protein interactions is required to rewire signaling circuitry in response to extracellular cues. Genome-wide analyses indicate that genetic variations such as polymorphisms account for the cellular plasticity difference among individuals. Our early genome-wide analyses of single nucleotide polymorphisms (SNPs) with potential influence on protein phosphorylation characteristics in human suggest that these SNPs may result in signal rewiring due to alteration of protein-protein interactions such as dimerization (Mol Cell Proteomics. 2010. 9, 623-34). Here we introduce a method for optically imaging intracellular protein interactions at nanometer spatial resolution in live cells using photoactivatable complementary fluorescent (PACF) proteins. Subsets of complementary fluorescent protein molecules were activated, localized, and then bleached followed by the assembly of super-resolution images from aggregate position of sum interactive molecules. Using microtubule plus-end hub protein EB1, we have established proof-of-concept and revealed previously uncharacterized EB1 structural determinant in tracking microtubule plus-ends. A combination of PACF with PhosSNP, a database we established for predicting SNPs that influence phosphorylation-elicited signaling rewiring enables us to visualize the mechanism of action underlying PhosSNP-elicited cellular plasticity changes. Thus, PACF provides a unique approach to visualize structural determinants underlying SNP-rewired spatial dynamics of protein-protein interactions at single molecule scale.

P1512**Board Number: B1013****From intracellular signaling to collective behavior: Understanding the dynamical origins of collective cAMP oscillations in *Dictyostelium discoideum*.**A. Sgro¹, D. Schwab¹, J. Noorbakhsh², P. Mehta², T. Gregor¹;¹Physics and Lewis-Sigler Institute, Princeton University, Princeton, NJ, ²Physics, Boston University, Boston, MA

Collective behaviors are a common feature of a diverse range of biological systems, from flocking birds and schooling fish to swarming bacterial colonies and embryonic morphogenesis. Such population-level behaviors in cellular collectives are controlled by complex biochemical signaling networks that reside within individual cells to coordinate cell-cell communication. One of the most striking examples of these behaviors is the cAMP-coordinated transition from an independent, single-celled state to a multicellular aggregate in the eukaryotic social amoebae *Dictyostelium discoideum*. However, describing how these population-level collective behaviors arise from intracellular signaling network dynamics is challenging. Even in well-studied model systems many of the dynamics of underlying signaling networks remain uncharacterized, complicating efforts to build a predictive model that takes into account each network component and interaction. Physical systems have taught us that collective behaviors do not depend on all the details of the system and that in regimes with behavioral changes only a few types of behaviors exist. Using these principles, we have condensed the amoeba's complex signaling network dynamics into a simple two-variable phenomenological model. We have confirmed our model's success at capturing these dynamics through quantitative experimental measurements, using microfluidics to control the extracellular environment and a FRET reporter for intracellular cAMP to monitor cellular responses. By using the single-cell model as a building block for a multicellular model, we are able to predict and experimentally verify novel population-level behaviors. Together, our model and experiments demonstrate that stochasticity is a key player both in the initiation and the ongoing coordination of collective behaviors, allowing cells to communicate in noisy extracellular environments. Our results lay the groundwork for using these types of models to identify common principles of how cellular collective cellular behaviors arise in nature.

P1513**Board Number: B1014****Mechanochemistry of persistent plasmid movement.**L. Hu¹, J. Liu¹;¹National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD

The segregation of DNA prior to cell division is essential to the faithful inheritance of the genetic materials. In many bacteria, the segregation of the low-copy-number plasmids involves an active partition system composed of ParA ATPase and DNA-binding ParB protein, which stimulates the hydrolysis activity of ParA. Both in vivo and in vitro experiments have shown that the ParA/ParB system

can drive persistent movement in a directed fashion, just like a processive motor protein. However, the underlying mechanism remains unknown. We have developed the first theoretical model on ParA/ParB-mediated motility. We establish that the coupling between the ParA/ParB biochemistry and its mechanical action works as a robust engine. It powers the directed movement of plasmids, buffering against diffusive motion. Our work thus sheds light on a new emergent phenomenon, in which elaborate mechanochemical couplings of non-motor proteins can work collectively to propel cargos to designated locations, an ingenious way shaped by evolution to cope with the lack of a processive motor protein in bacteria.

P1514

Board Number: B1015

Mechanistic and Structural Insight into Protein Liquid Droplets Formed by the DEAD-Box Helicase LAF-1.

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Phase transitions into liquid and hydrogel states have emerged as playing critical roles in cellular organization, including the regulation of RNA/protein assemblies, and their dysregulation in disease. However, the underlying molecular mechanisms remain poorly understood. Here, we show that LAF-1, a DEAD-box RNA helicase associated with P granules in the *C. elegans* germline, can phase separate into liquid droplets in-vitro, at near physiological (low μM) concentrations. LAF-1 droplet formation is driven by its N-terminal RGG domain, which is predicted to be disordered. We map the phase diagram of LAF-1, demonstrating that salt concentration finely tunes droplet propensity. Furthermore, we use microrheology and FRAP to reveal the local viscoelastic properties and molecular dynamics of droplets as a function of salt and time. These findings provide mechanistic and structural insight into the phase transition-driven assembly of liquid-like intracellular organelles.

P1515

Board Number: B1016

MOLECULAR BIOPHYSICAL CHARACTERIZATION OF Krr1p-ssDNA COMPLEX.

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Many syndromes are specifically related to defects in interactions between proteins and DNA. The study of the interaction between Krr1 with DNA will improve our understanding of fragile X syndrome and provide the basis to have the knowledge of regulatory functions within the nucleus. We studied the GXXGloop within the Krr1 KH domain specifically K159RRQRLIGPKGSTLKALELLTNCW182 (Krr1p) using Circular Dichroism (CD), Fourier transform Infrared Spectroscopy (FT-IR), and Two Dimensional Infrared

Spectroscopy (2DIR) to establish the secondary structure composition and molecular behavior of the Krr1p upon thermal denaturation. Krr1p was observed to have both α -helical and random coil components. In addition, the thermal transition temperature (T_m) was determined. We then proceeded to study the Krr1p-ssDNA interaction using CD and FT-IR which resulted the destabilization of Krr1p within the complex as the temperature increased above 40°C. More importantly the molecular dynamic of the complex will be discussed.

P1516

Board Number: B1017

Microscopic (FLIM) analysis of NADH state changes during cell cycle progression in cancer cells.

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Cancers exhibit high metabolic activity via the “Warburg effect”, the shift to a high rate of glycolysis followed by lactic acid fermentation in the cytosol, rather than a low glycolytic rate followed by pyruvate oxidation. This increased glycolytic state causes a corresponding increase in cytosolic free NADH production. Increased NADH production in cancer cells is also the result of a heightened metabolic demand due to rapid cell division. Thus, NADH levels are indicative of cancer cell metabolic state. Specifically, a higher free/bound NADH ratio represents higher glycolytic activity, whereas a lower free/bound NADH ratio represents higher mitochondrial respiration. Without knowledge of NADH state (free or bound), NADH levels cannot be effectively studied. However, little is known about NADH state or distribution in cancer cells. Phasor-Fluorescence Lifetime Imaging Microscopy (Phasor-FLIM) is a useful tool to study NADH state and distribution, but NADH state and distribution are variable from cell to cell. NADH state analysis at different cell-cycle stages will allow for more specific and detailed comparisons between cancer cells. Here, Phasor-FLIM is used to quantify changes in NADH state and distribution during cell cycle progression. Different cancer cell lines, including Mb231 and U2OS, are used. Cancer cells in their flat, adherent interphase stage exhibit a low free/bound NADH ratio. During their rounded mitotic stage, cancer cells transition to a high free/bound NADH ratio and then return to a low free/bound NADH ratio as they divide. Therefore, striking change in NADH state during part of mitosis is observed. This suggests that cancer cells are at a high mitochondrial respiratory state in other stages but exhibit a spike in glycolysis during mitosis, returning to their original state by the time of division. The mitotic stages at which the spike occurs will be determined using mitosis specific fluorescent markers, and measurements will be compared to those in noncancerous cells.

P1517**Board Number: B1018****Getting in shape: mechanics of the extracellular matrix and *Drosophila* follicle geometry.**I. Heemskerk¹, S. Streichan¹;¹Kavli Institute for Theoretical Physics, Univ California-Santa Barbara, Santa Barbara, CA

During morphogenesis, genetics and mechanics determine tissue shape by controlling growth, movement, and shape change of cells. This involves forces that can be exerted within cells by regulation of the cytoskeleton, or externally by the extracellular matrix. The *Drosophila* follicle develops from round to asymmetrically elongated. We hypothesize that the basal membrane, which becomes organized during elongation, exerts the dominant forces required for this shape change. To guide our intuition and refine this idea, we formulate a mathematical model for follicle mechanics. Testing this requires organ scale or in toto data. Few analysis tools for effectively analyzing in toto image data are available. We present ImSAnE, a general software tool for quantitative analysis on curved tissue surfaces. We use it to quantify follicle geometry, infer tension anisotropies in the follicle epithelium, and measure the structure of the basement membrane. We then test our model against these data.

P1518**Board Number: B1019****Limitations of the limiting-pool mechanism for controlling the size of biological structures in cells.**L. Mohapatra¹, T. Lagny², J. Kondev¹;¹Department of Physics, Brandeis University, Waltham, MA, ²Institut Curie, Paris, France

Cells contain a number of micron-scale structures whose physiological functions are related to their size. Examples include mitotic spindles, cilia, and actin cables. Each of these structures is characterized by a narrow size distribution and they are composed of molecular building blocks that diffuse in the cytoplasm. A key question in cell biology is how the size of these structures is maintained in light of constant turnover of their molecular components. A related question is how different sized structures can coexist in the same cytoplasm while making use of the same building blocks. The role of the finite pool of building blocks in the control of size has received considerable attention as it offers a simple mechanism of size regulation: the size of the structure grows until the pool of available building blocks is depleted. Using theory and simulations, we study the limiting-pool mechanism of size control. We consider a simple model where a structure grows from a nucleating center by addition of diffusible building blocks which can also dissociate. The number of building blocks in the cell is fixed. While the growth rate is proportional to the free monomer concentration, we assume the dissociation rate to be constant. When we consider a single structure, we find that the distribution of sizes in steady state is peaked around a mean value and therefore the limiting pool of components provides a size-regulation

mechanism. Surprisingly, if there are two identical nucleating centers we find that the total mass of the two generated structures reaches a stable value, however their individual sizes are characterized by large fluctuations. When considering two competing nucleating centers with different rates of assembly, the one with the higher rate wins and produces a structure of a well-defined size, while the other structure does not form. Our theoretical study illustrates the limitations of the limiting-pool mechanism of size control and highlights the importance of additional mechanisms that are required if the cell is to generate multiple structures of a well-defined size using a common pool of building blocks.

P1519

Board Number: B1020

Investigation of the electrical properties of doped melanin.

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Melanin is a multifunctional pigment found in many species of animals. Its best understood function is the absorption of UV radiation in the human skin. Although the synthesis pathways of melanin are known, the final molecular structure of natural melanin remains controversial. It has been observed that the melanin molecules have the ability to incorporate water and metallic ions into their structure, thereby affecting behavior. Melanin has been reported to have thermoelectric, piezoelectric, and semiconducting properties in addition to its known biological functions.

This study focuses on the change in electrical resistance of melanin with the addition of several dopants. The melanin was extracted from a natural source, *Sepia officinalis* (cuttlefish) ink, centrifuged at 2900G, and sealed into glass samples with copper leads on both ends. The melanin was tested with several dopants: copper sulfate, zinc sulfate, ferrous sulfate, calcium chloride, lithium chloride, clay, and silicified cellulose. The dopants were added to the extracted melanin by heating the 50:50 mixtures of melanin and dopant for four hours with constant stirring. The excess dopant was then centrifuged out and the doped melanin was made into samples, each containing approximately 2 grams of the mixture. A multimeter was used to measure resistance over a period of 10 minutes.

Compared to the control (undoped) melanin, the only dopant that showed a substantial decrease in resistance was copper sulfate, having a decrease in resistance of about one order of magnitude. Additional testing has shown that increased external temperature during testing also decreases the resistance of the samples. This study indicates that the addition of the copper sulfate can significantly decrease the resistance of natural melanin. This may enhance its potential for use in electrical applications.

P1520**Board Number: B1021****Evaluation of temperature dependent electrical properties for hydrated natural melanins.**H. Li¹, E. Mirizio^{2,3}, S. Rajasimhan², B. Qian¹, S. Baranowitz², M.F. Kiani⁴, F. Ren⁴;¹Mechanical Engineering, Temple University, Philadelphia, PA, ²EpiTek, Medford, NJ, ³College of Biomedical Engineering, Drexel University, Philadelphia, PA, ⁴College of Engineering, Temple University, Philadelphia, PA

Melanins are widespread pigments found in cells called melanophores or melanocytes. Though known primarily for their ability to absorb ultraviolet light, melanins have a variety of interesting physical and electrical properties including photoconductivity, piezoelectricity, and thermoelectricity. These less studied properties may be related to some important biological functions. For example, thermoelectricity could be utilized to achieve cooling as a result of electrical current induced heat pumping.

Many studies on the thermoelectric properties of melanins were performed in the lyophilized (dehydrated) state, which is very different from the physiological state in which melanins exist in vivo. Since the electrical properties of melanins are strongly affected by their hydration level, this study examined melanins in their naturally hydrated states. Sepia melanin was centrifuged, washed, and then placed in sealed capsules. Polarization, electrical conductivity, heat induced voltage change, and thermal conductivity were measured.

Our results showed that natural melanins in their hydrated states exhibited natural polarization, which saturated over time. When subject to heating, voltage across the sample changed as a function of temperature difference. Both increasing and decreasing trends were observed, and further studies are desired to reveal the underlying mechanisms of the heat-induced voltage change. Nevertheless, based on our preliminary study, we suspect that the temperature dependent electrical properties are related to the pyroelectric phenomena of melanins where the polarization is a function of the temperature.

Results of the current study not only provide information on the thermal electrical properties of melanins, which are the basic pigment molecules of melanocytes, but also provide a general methodology to study the electrochemical behavior of pigments of many types that may be essential to key cellular processes.

Engineering Tissues and Organs

P1521

Board Number: B1022

Engineered Cell Penetrating Peptides for Stem Cell Biology Control.

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Cell Penetrating Peptides (CPPs) are utilized in various molecular delivery applications. CPPs actively transport into mammalian cells across the plasma membrane barrier. CPPs are commonly derived from proteins such as the HIV-1 transcriptional activator and the murine vascular endothelial cadherin. The lengths of CPPs generally range from 30-50 amino acids and can lead to preparative challenges. In this presentation, we will introduce a newly engineered short cell penetrating peptide (herein PepB; Due to patent filing issues we are not able to disclose the PepB sequence at this time) as an enabling platform technology. We have currently reduced the CPP sequence to PepB (<10mer). We have tested the PepB sequence to deliver bioactive molecules to mammalian cells and they have increased the mode of action inside stem cells when repeat sequences were utilized. Experimentally, PepB molecules were cultured with human embryonic kidney, lung cancer and neural stem cells and tested over multiple days to assess toxicity and biological activity (e.g., neuronal differentiation). Cellular uptake propensities were modulated based on PepB's covalent attachment to select small molecules. In order to more precisely define PepB's cell penetrating properties, we conducted live-cell imaging and confirmed that PepB-incorporating synthetic agents can enter cells and label them for multiple months in vitro.

P1522

Board Number: B1023

Silencing the transcriptional repressor ZCT1 alters alkaloid biosynthesis in hairy root cultures of *Catharanthus roseus*/.

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The medicinal plant, *Catharanthus roseus*, is the source of pharmaceutically valuable anticancer alkaloids, vincristine (VCR) and vinblastine (VBL). These alkaloids are produced only in *C. roseus* and at extremely low levels (0.0002 wt%). Due to their low levels, the isolation of these compounds is both laborious and costly, ranging from \$4 to \$60 million/kg. Despite these barriers, these alkaloids have been effectively used to treat cancer for over 50 years.

The biosynthesis of VCR and VBL is not entirely known but involves at least 35 intermediates and 30 enzymes. In an effort to increase VCR and VBL, key enzymes in the *C. roseus* biosynthetic pathway have

been overexpressed but this strategy has proven unsuccessful since the enzymatic bottleneck is shifted downstream. However, transcription factors regulate *multiple* genes in the pathway and therefore engineering their expression may potentially increase the overall flux through the network and lead to increased production of the desired compounds.

Here, we study key transcription factors involved in regulating multiple alkaloid biosynthetic enzymes. Since ZCT1 (Zinc finger *Catharanthus* transcription factor) is a known repressor of alkaloid biosynthetic enzymes, we established stable *C. roseus* hairy root cultures with estrogen-inducible *Zct1*-silencing through RNA interference (RNAi). The results of silencing this key repressor and implications in alkaloid biosynthesis in *C. roseus* were studied. Levels of *Zct1* decrease with estradiol treatment, and importantly, *Zct1* induction with plant hormone methyl jasmonate is overcome by estradiol silencing. *Zct1*-silencing also leads to an increase in specific metabolites including new metabolites not observed in the wild-type hairy root cultures. These changes in metabolites may potentially be correlated with changes in the regulation of downstream alkaloid biosynthetic genes.

P1523

Board Number: B1024

Step-wise Assembly of Membrane Anchored Oligonucleotides for the Synthesis of Three-Dimensional Organoids.

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There is growing interest in using extracellular matrix supported 3D organoid cultures to model cell-cell and cell-matrix interactions. Compared to 2D cultures, these organoid models are better in-vitro representations of disease and more faithfully recapitulate in-vivo phenotypes such as gene expression patterns, toxicology profiles and cell signaling. However, current 3D culture methods are limited in their ability to rapidly and reproducibly generate heterotypic assemblies of controlled size and shape, particularly when using primary cells. DNA-programmed assembly is one approach for controlling cell-cell or cell-surface adhesion without the need to genetically modify cells. We report a new strategy utilizing stepwise assembly of fatty acid modified oligonucleotides to label the phospholipid membrane of cells with single stranded DNA. This DNA is subsequently used as a reactive handle to control adhesion by specific duplex formation. Here we demonstrate proof of concept experiments for this strategy and robust DNA labeling of several primary cell samples. In addition, we use this labeling strategy in conjunction with a capping strand and microwells to rapidly synthesize aggregates of cells in desired sizes and shapes beyond simple spheroids. We demonstrate that these aggregates can be

transferred to extracellular matrix for further culture and study. We anticipate that simplifying the synthesis of microtissues will increase the accessibility of 3D organoid cultures as model systems.

P1524

Board Number: B1025

Differential signaling through the EGFR pathway drives distinct collective cell behaviors in epithelial tissues.

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Emergent properties of tissues arise from the collective behavior of their constituent cells. These coordinated behaviors are believed to result in part from controlled or stochastic cell-to-cell variation in signaling and play important roles in development, homeostasis and disease processes such as invasion and metastasis. We previously demonstrated that heterogeneous expression of oncogenic Ras among mammary epithelial cells was sufficient to elicit multiple and distinct collective behaviors. Along with normal morphogenesis, behaviors observed in these tissues included cell extrusions, collective cell migration, and single hypermotile cells. Interestingly, emergent behaviors occurred with very low frequency when all cells of the tissue expressed similar levels of oncogenic Ras and depended on signals propagated through EGFR, Ras/MAPK, and PI3K. However, it is unknown how heterogeneous groups of cells decode these signals to make a collective behavioral decision. We used siRNA to target these key signaling pathways in high- and low-Ras expressing cells in order to determine whether some or all cells in a group require these signals to promote each emergent behavior. We find that in general, elevated pathway activation is required in a single 'leader' cell within the group for cell extrusion and collective cell migration behaviors. However, a counterintuitive requirement for signaling in the 'follower' cells also exists, and this regulates the shift between one emergent behavior and another. Indeed, some loss-of-function perturbations in the 'follower' cells actually accentuated emergent behaviors within the tissue. These studies imply that therapeutics delivered to a heterogeneous tumor environment could lead to unexpected enhancement of malignant behaviors.

P1525**Board Number: B1026****Development of a Novel, Physiologically and Anatomically Realistic *in vitro* Pediatric Blood Brain Barrier on a Chip.**S.P. Deosarkar¹, B. Augelli², B. Prabhakarandian³, B. Krynska², M.F. Kiani⁴;¹Department of Mechanical Engineering, Temple University (PA), Philadelphia, PA, ²Department of Neurology and Shriners Hospitals Pediatric Research Center, Temple University, Philadelphia, PA,³Biomedical Technology, CFD Research Corporation, Huntsville, AL, ⁴College of Engineering, Temple University, Philadelphia, PA

Blood brain barrier (BBB) is a physical and functional barrier that protects and regulates the brain parenchyma. Thus, a dysfunctional BBB can lead to various neurological disorders. Pediatric and adult BBB exhibit significant differences in its structure and function. In the immature brain, major risk factors of brain damage such as inflammation and/or hypoxia/ischemia have been shown to result in increased BBB permeability and are involved in the development of diseases such as cerebral palsy and traumatic brain injury. However, the biological mechanisms underlying the pathological changes of blood-brain barrier permeability in children are still largely unknown. This is primarily because of the ethical difficulties of conducting experiments in children and the lack of good experimental models of pediatric BBB. Thus, the objective of this study was to develop a physiologically realistic *in vitro* pediatric BBB model on a chip (B³C) that could be used to study the biological mechanisms of pediatric BBB function and/or dysfunction. We fabricated a B³C using an optically clear, oxygen permeable polymer, polydimethylsiloxane (PDMS) on a glass slide with vascular (apical) and tissue (basolateral) compartments. We isolated rat brain endothelial cells (RBECs) from rat pups. RBECs were cultured in the vascular compartment of the device under static or flow conditions in the presence or absence of astrocyte conditioned media (ACM). The barrier formation by RBECs in B³C was assessed by immunofluorescence staining of RBECs for tight junction molecules [e.g. zonula occludens-1 (ZO-1)] and by measuring transendothelial electrical resistance (TEER) and permeability of fluorescently tagged molecules of different sizes (e.g. sodium fluorescein and FITC-Dextrans of different molecular sizes) across the barrier formed by RBEC. Fluorescence microscopy was used to analyze the permeability of fluorescently tagged molecules across the *in vitro* BBB in our B³C model. We observed significant differences in the permeability of different sized molecules (e.g. sodium fluorescein and FITC-Dextrans of different molecular sizes). Additionally, we observed that RBEC exhibited more uniform tight junction formation when cultured under flow conditions in B³C compared to static conditions. Thus, in this study, we have developed a first realistic pediatric BBB model (B³C) using RBECs from rat pups which could serve as an *in vitro* model system for studying BBB function in pediatric neurological diseases as well as for testing novel therapies for these diseases.

P1526**Board Number: B1027****Contractility mediated wound closure in mesenchymal 3D microtissues.**J. Eyckmans^{1,2}, M.S. Sakar³, V. Shenoy⁴, D. Eberli⁵, B. Nelson³, C.S. Chen^{1,2};

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Wound healing after injury is a highly dynamic process requiring migration and proliferation of multiple cell types. Many insights on the cellular interactions and signaling networks underlying wound healing are based on healing of scratches in monolayers of epithelial cells in 2D culture systems or wounds in *Drosophila* embryos. In contrast, how fibroblasts participate in wound healing is less well understood. Unlike epithelial cells lining surfaces and lumen *in vivo*, fibroblasts are ensconced in fibrous ECM and therefore don't display apical-basal polarity. Hence, the influx of cells into the site of injured ECM, the closure of the wound, and the subsequent ECM stiffening are all components of the process that are not captured by planar *in vitro* models for wound healing. To overcome this limitation, we present in this work a novel wound healing model to evaluate healing of microsurgically-induced wounds in an engineered 3D microtissue of fibroblasts embedded in a type I collagen matrix. Using our microfabricated tissue gauge technology (1), we generated arrays of 3D microtissues suspended between flexible cantilevers that simultaneously constrain the microtissues and report microtissue contractility in real-time. After compaction, microtissues were damaged with a microsurgical knife mounted to a microrobotic manipulation platform. Depending on the size of the incision (longitudinal axis: 100 to 300 μm), the wound closed within 12 to 24h. Gap closure was associated with progressively increasing microtissue contractility concomitant with directed migration of the cells towards filling of the defect. While phosphorylated myosin was detected with immunohistochemistry during closure, no purse string was observed. Using pharmacologic reagents, we found that the mechanisms for closure in this 3D model were distinct from those involved in healing of traditional 2D scratch wounds. Both increased and decreased Rho signaling reduced healing rates in 3D, but had no effect in the 2D scratch assay. In contrast, Cdc42 or Rac1 inhibitors did not impact 3D closure but prevented wound closure in 2D. We will present additional findings elucidating and further validating the mechanisms involved in driving wound healing in this model. These studies introduce a new model for studying wound, and highlight important mechanistic differences between gap closure by fibroblasts embedded in 3D matrix versus adherent to a planar surface.

Tissue Development and Morphogenesis 2

P1527

Board Number: B1029

Effects of exogenous retinoic acid on regenerating intestines from sea cucumbers in organ culture.

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The study of the mechanisms that allow the spontaneous regeneration of lost complex organs in simpler organisms might provide clues on how to stimulate organ regeneration in humans. The sea cucumber *Holothuria glaberrima* exhibits the extraordinary capacity to regenerate its gastrointestinal tract in less than four weeks after induced evisceration. Previously, our group has shown that some components of the retinoic acid (RA) signaling pathway, homologous to the chordate counterparts, are expressed during intestinal regeneration in sea cucumbers. However, the role of the RA signaling pathway during regeneration in sea cucumbers is still poorly understood. Here, we investigated the effects of exogenous retinoic acid (RA) during the intestinal regeneration of sea cucumbers using an *in vitro* approach. Regenerating gut rudiments were dissected from sea cucumbers at 7 days post-evisceration (dpe) and cultured for 60 h in modified L-15 cell culture medium containing RA at different doses (100, 50, 25, or 2.5 μ M). Gut rudiments incubated with DMSO were used as a control group. BrdU (50 μ M) was added to the culture medium for the last 24 h of culture to evaluate the effect of RA on cell proliferation. Subsequently, the intestines were processed according to the immunocytochemical techniques previously used in our lab. The size of the regenerating gut rudiment, the percentage of the proliferating cells, and the presence of muscle fibers or spindle-like structures (a measure of muscle dedifferentiation as cell condense their myofilaments) in the regenerating gut rudiment and adjacent mesentery were evaluated in tissue sections using fluorescence microscopy and the software Image J. We observed an increased gut rudiment's size in the tissues treated with the highest doses of RA (100 and 50 μ M) compared with those treated with the lowest doses of RA (25 and 2.5 μ M) and the control. In contrast, the percentage of dividing cells decreased with the RA treatment in a dose-dependent manner, from 15% for the control group (DMSO) to 5% in the group treated with RA 100 μ M. Unexpectedly, short muscle fibers were observed in the mesothelium of the regenerating gut rudiments treated with RA and in the control group. However, the fibers were more abundant in tissues treated with RA 2.5 μ M. At 7 dpe muscle fibers are not normally observed in the gut rudiment since they disappear at early regenerative stages and reform later on during regeneration. Taken together, these results suggest that the exogenous RA may accelerate the intestinal regeneration in sea cucumbers. The molecular mechanisms by which RA affects the intestinal regeneration will be explored in future experiments.

P1528**Board Number: B1030****c-Jun N Terminal Kinase (JNK) is required for archenteron invagination in the developing sea urchin embryo.**J. Long¹, M.T. Peeler¹;¹Biology, Susquehanna Univ, Selinsgrove, PA

The cellular events of gastrulation have been well characterized in sea urchins; however the underlying molecular mechanisms have not been well studied. This study seeks to understand the role of c-Jun N-terminal kinase (JNK), a component of the planar cell polarity pathway in the development of sea urchins. JNK has been shown to be required for embryonic development in other species, particularly in mediating cell migration. Adenosine triphosphate competitive JNK inhibitor SP600125 was used to characterize JNK's role in sea urchin embryos. We determined that loss of JNK function specifically disrupts invagination of the archenteron but not other cellular movements. Embryos treated with 2uM SP600125 developed normal vegetal plates but did not invaginate to form an archenteron. Other notable embryonic cell movements, specifically skeletogenic mesenchyme ingression, were not affected with treatment. Despite lacking a visible archenteron, cells at the vegetal plate expressed molecular markers for endoderm differentiation, such as alkaline phosphatase staining. These results suggest that JNK is required for invagination and morphogenesis of the archenteron but not for differentiation of endoderm, pigment, or skeletogenic mesenchyme cells, during sea urchin gastrulation.

P1529**Board Number: B1031****A force balance explains local and global cell movements during early zebrafish development.**J. Chai¹, A.L. Hamilton², C.D. Buckley¹, M. Krieg², I.H. Riedel-Kruse³, A.R. Dunn¹;¹Chemical Engineering, Stanford University, Stanford, CA, ²Molecular and Cellular Physiology, Stanford University, Stanford, CA, ³Bioengineering, Stanford University, Stanford, CA

Biological tissues undergo radical changes in shape during development, growth, and repair. Little is known about how 100-1000s of cells generate these intricately choreographed transformations. Here we examine the origins of collective cell movement during early zebrafish development, a powerful model system for addressing this topic. The first morphogenetic movement in zebrafish is epiboly, in which the blastoderm spreads uniformly across the yolk and converges at the vegetal pole. In this study, we show that tension within the actin band, a dense mesh of actin and myosin that assembles near the blastoderm margin, coordinates blastoderm migration on both the organismal and cellular length scales. We find that gentle mechanical deformation of the developing embryo causes shape-dependent alterations in blastoderm migration. In addition, we observe realignment of the anterior-posterior (AP) axis away from the animal-vegetal axis toward the new long axis of the embryo. Chemical disruption of

the actin band restores uniform blastoderm migration, eliminates AP axis reorientation, and increases cellular disorder at the blastoderm margin. An analytical model and resulting simulation based upon forces generated by the actin band recapitulates the experimental observations. Taken together, our findings suggest that a relatively simple physical mechanism, in this case tension generated by the actin band, can lead to long-range coordination of cell movements that would be difficult to achieve by biochemical signaling alone. Further, we show that AP axis specification, a fundamental step in the development of all embryos, is exquisitely sensitive to mechanical cues. These observations support the possibility that mechanical forces may play a ubiquitous role in

P1530

Board Number: B1032

Development of the Zebrafish Pronephros: Cellular and Genetic Analysis of Nephron Tubulogenesis and Segment Patterning.

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Chronic kidney disease (CKD) affects 300 million people worldwide, representing the 12th leading cause of death. Understanding how nephrons, the simplest functional unit of the kidney, develop can lend insight into the processes that go awry during disease. The zebrafish pronephros provides an excellent *in vivo* system to study the mechanisms of vertebrate nephrogenesis. When and how renal progenitors in the zebrafish embryo undergo tubulogenesis to form nephrons is poorly understood, but is known to involve a mesenchymal to epithelial transition (MET) and the acquisition of polarity. We determined the precise timing of these events in pronephros tubulogenesis. As the ternary polarity complex is an essential regulator of epithelial cell polarity across tissues, we performed gene knockdown studies to assess the roles of the related factors atypical protein kinase C iota and zeta (*prkci*, *prkcζ*). We found that *prkci* and *prkcζ* serve partially redundant functions to establish pronephros tubule epithelium polarity. Further, the loss of *prkci* or the combined knockdown of *prkci/ζ* disrupted proximal tubule morphogenesis and podocyte migration due to cardiac defects that prevented normal fluid flow to the kidney. Surprisingly, tubule cells in *prkci/ζ* morphants displayed ectopic expression of the transcription factor *pax2a* and the podocyte-associated genes *wt1a*, *wt1b*, and *podxl*, suggesting that *prkci/ζ* are needed to maintain renal epithelial identity. Knockdown of genes essential for cardiac contractility and vascular flow to the kidney, such as *tnnt2a*, or elimination of pronephros fluid output through knockdown of the intraflagellar transport gene *ift88*, was not associated with ectopic pronephros gene expression, thus suggesting a unique role for *prkci/ζ* in maintaining tubule epithelial identity separate from the consequence of disruptions to renal fluid flow. Interestingly, knockdown of *pax2a*, but not *wt1a*, was sufficient to rescue ectopic tubule gene expression in *prkci/ζ* morphants. These data suggest a model in which the redundant activities of *prkci* and *prkcζ* are essential to establish tubule epithelial polarity and also serve to maintain proper epithelial cell type identity in the tubule by inhibiting *pax2a* expression. Intriguingly, segment patterning of the pronephros was initially intact in *prkci/ζ* morphant

embryos suggesting the overarching MET and nephron patterning processes occur independent of one another. To better understand the conserved segmentation process of the nephron a forward genetic screen was performed. From our screen candidate mutations that disrupt the epithelial segments of the pronephros have been identified. These studies provide a valuable foundation for further analysis of MET during nephrogenesis, and have implications for understanding the pathways that affect nephron epithelial cells during kidney disease and regeneration.

P1531

Board Number: B1033

Genetic Analysis and Characterization of the Novel Zebrafish Podocyte Mutant *zeppelin*.

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The zebrafish pronephros is highly conserved with higher vertebrates, including mammals, thus making it an excellent model to study kidney formation. The nephron, or functional unit of the kidney, modifies the blood to excrete metabolic waste using a blood filter with specialized epithelial cells known as podocytes. Knowledge about podocyte development is highly relevant to the treatment and prevention of kidney disease, as podocyte injury leads to progressive scarring and nephron atrophy culminating with end stage renal failure, however the pathways that specify the podocyte lineage remain poorly understood. Through an edema based F3 forward genetic screen, we isolated *zeppelin* (*zep*), which displays edema at 5 days post fertilization and forms severely reduced podocytes as assayed by *in situ* hybridization with markers such as *wt1a*, *wt1b*, *lhx1a*, and *nephrin*. *zep* mutant embryos are unaffected by retinoic acid (RA) treatment, indicating that RA acts upstream or in an unrelated pathway with that of *zep*. Interestingly, the interrenal gland of *zep* mutants is increased in size. Preliminary cell death and proliferation assays in *zep* mutants did not show any alterations from wildtypes, suggesting the possibility of a cell fate switch between the podocyte and interrenal lineages. To determine the genetic lesion responsible for *zep*, we utilized a combinatorial strategy of whole genome sequencing (WGS) and meiotic mapping. These techniques narrowed the region to a small interval on chromosome 15, and candidate genes were knocked down with morpholinos (MO). Three independent MOs designed against *breast cancer 2, early onset* (*brca2*) phenocopied *zep*, recapitulating both the unique late edema phenotype, as well as the vast reduction of podocytes. This suggests, for the first time, that *brca2* is essential for renal development. Taken together, these findings provide novel insights into the genetic regulatory networks that control podocyte formation in the vertebrate kidney

P1532**Board Number: B1034****NADPH oxidase activity is required for axonal development in zebrafish.**C.J. Weaver¹, Y.F. Leung¹, D.M. Suter¹;¹Department of Biological Sciences, Purdue University, West Lafayette, IN

Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases produce bursts of reactive oxygen species (ROS). Long thought to be involved exclusively in host defense, NADPH oxidase-derived ROS are now appreciated as critical signaling intermediates in a growing number of cell signaling pathways. However, their role in axonal growth and guidance has not been extensively studied. The zebrafish (*Danio rerio*), because of excellent live-imaging capability and well-developed molecular genetics, is an extremely powerful model system for studying axonal development *in vivo*. Our current study is the first to detail the expression of the known zebrafish NADPH oxidase (Nox) isoforms, Nox1, Nox2, Nox5 and Duox during development. Using quantitative PCR, we show that all Nox isoforms are expressed during the first two days of development. Our results indicate that expression levels differ greatly among Nox isoforms and over time. We used *in situ* hybridization to investigate Nox isoform expression in the brain, retina, and spinal cord. In support of the qPCR results, we detected expression of all four Nox isoforms in all brain tissues. Surprisingly, individual Nox isoforms are not tissue specific, but expressed broadly throughout the brain and spinal cord. We next investigated the functional significance of NADPH oxidases in axonal growth and guidance by applying two broad-spectrum Nox inhibitors, VAS2870 and Celastrol, during critical periods of axonal development. Both the anterior commissure and the optic nerve showed diminished axonal growth and the formation of mistargeted axons when NADPH oxidases were inhibited. This demonstrates that despite broad expression, inhibition of NADPH oxidases only interferes with the development of specific axons. In order to explore the function of individual Nox isoforms during axonal development, we have used a CRISPR/Cas9-based method to generate the first specific loss-of-function mutants for Nox1, Nox2, Nox5 and Duox in zebrafish. Upon the completion of genetic and phenotypic analysis, we will assess axonal growth and guidance in each mutant line. This study is the first to demonstrate an *in vivo* role for NADPH oxidase-derived ROS in axonal development.

P1533**Board Number: B1035****Oriented cell divisions during collective cell migration in *Drosophila*.**L.K. Lewellyn¹, V. Sun², S. Horne-Badovinac²;¹Biological Sciences, Butler University, Indianapolis, IN, ²MGCB, University of Chicago, Chicago, IL

Morphogenesis is a highly dynamic process that is critical to determine the final shape of an organ. We use *Drosophila* egg development as a highly tractable model system to study morphogenesis. Each fly egg develops from a multicellular, organ-like structure called an egg chamber. The egg chamber is composed of a central germ cell cluster surrounded by a layer of epithelial cells, called the follicle cells. As it grows during the 14 developmental stages of oogenesis, the egg chamber elongates preferentially

along the anterior-posterior (A-P) axis to generate an elliptical egg. During the early stages of egg chamber elongation, the follicle cells undergo a collective migration around the circumference of the egg chamber, resulting in a global tissue-level rotation. Collective follicle cell migration leads to the remodeling of the underlying basement membrane, which is thought to create a molecular corselet that promotes preferential growth of the egg chamber along the A-P axis. Through stage 6 of oogenesis, the follicle cells are undergoing both collective migration and mitosis. Because oriented cell divisions contribute to tissue elongation in other contexts, we asked whether oriented follicle cell divisions contribute to egg chamber elongation. Measuring the division angle of mitotic follicle cells demonstrated that there is an increasing bias in the orientation of division, such that as the egg chamber develops, follicle cells preferentially divide closer to the A-P axis. At the end of stage 6, the follicle cells stop dividing and switch to endocycling; however, collective migration continues through stage 8. If we prolong mitoses into these later migratory stages, we observe a significant increase in elongation of the egg chamber. Further, blocking follicle cell migration disrupts the bias in the division angle, suggesting that collective migration provides a cue to orient the dividing cells along the A-P axis. Therefore, our work suggests that there is a bias in the orientation of divisions in the developing egg chamber, and that the ability of cells to collectively migrate correlates with this progressive bias.

P1534

Board Number: B1036

Cell cycle arrest is required for cell invasive behavior.

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Despite its critical role in development, tissue homeostasis and cancer, the molecular mechanisms that drive cell invasive behavior are poorly understood. We are utilizing *Caenorhabditis elegans* anchor cell (AC) invasion as an *in vivo* model to identify transcription factors required to confer an invasive phenotype. Through a large-scale RNAi screen we have identified cell cycle arrest as a new transcriptionally-mediated feature of the invasion program. The conserved nuclear hormone receptor, *nhr-67/tlx* maintains the AC in a G1 arrested state through upregulation of *cki-1* (p27/KIP1) expression. Depletion of *nhr-67* creates non-invasive, mitotic ACs that fail to generate invadopodia or express matrix metalloproteinases (MMPs). Induced AC-specific expression of *cki-1* in *nhr-67* mutants restored G1 arrest, invadopodia formation, pro-invasive gene expression and rescued invasion. Together, our data indicates the requirement for a genetic program that links cell cycle arrest to the differentiation of the invasive phenotype and provide a possible explanation for the paradoxical observation that many metastatic cancers are non-proliferative.

P1535**Board Number: B1037****Non-muscle myosin regulates *C. elegans* epidermal morphogenesis.**K. Mastronardi¹, D. Wernike¹, Y. Chen¹, A.J. Piekny¹;¹Department of Biology, Concordia University, Montreal, QC

Tissue formation requires coordinated cell migration and shape changes, and is essential for the development of metazoans. However, it is challenging to study in complex organisms. We study epidermal morphogenesis in *C. elegans*, which is an excellent model due to its simplicity and amenability to microscopy, genetics and transgenic tools. Furthermore, the underlying processes that govern migration and cell shape changes are highly conserved among metazoans. We are interested in ventral enclosure, when the ventral epidermal cells migrate over underlying neuronal precursor cells, the neuroblasts, in order to encase the embryo in a single layer of epidermis. This process is initiated by the migration and adhesion of two pairs of anterior leading epidermal cells, followed by the migration and adhesion of eight pairs of posterior pocket epidermal cells. Previous studies showed that migration of the ventral epidermal cells requires the formation of F-actin-rich protrusions, and genes that mediate their formation are essential for ventral enclosure. However, non-muscle myosin has not been studied in this process, even though it is a key regulator of cell migration and shape change in other organisms. Furthermore, it is not clear how the underlying neuroblasts contribute to ventral enclosure; they likely secrete chemical cues that regulate migration of the ventral epidermal cells, but also could provide mechanical cues.

We found that the Rho GEF Ect2 (*ect-2* in *C. elegans*) is required for ventral enclosure. In *ect-2* mutant embryos, migration of the ventral epidermal cells and neuroblasts are strongly delayed. RhoA is also required for ventral enclosure, and both RhoA and ECT-2 are expressed in epidermal cells and neuroblasts. Since RhoA regulates Rho kinase to mediate non-muscle myosin contractility for migration in other organisms, we propose that myosin also regulates ventral epidermal cell migration for ventral enclosure. In support of this hypothesis, we found that GFP-tagged non-muscle myosin localizes to foci that organize into a supra-cellular ring-like structure around the ventral pocket cells during ventral enclosure. Surprisingly, these foci come from both epidermal cells and neuroblasts, and we found that the number and intensity of foci in both cell types are reduced in embryos mutant for *ect-2* or Rho kinase. We are currently examining the tissue-specific contribution of non-muscle myosin during ventral enclosure. These studies should shed light on the interplay between tissues during embryonic development.

P1536**Board Number: B1038****NAD⁺ Deficiency Triggers Changes in Metabolic Energy Pathways Affecting Gonad Development in *C. elegans*.**M.R. McReynolds¹, W. Wang¹, M. Shu¹, W. Hanna-Rose²;¹Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA, ²Pennsylvania State University, University Park, PA

NAD⁺ is an essential co-enzyme necessary for electron transport in many metabolic reactions, such as the citric acid cycle, glycolysis and the regeneration of ATP from ADP. NAD⁺ functions as a substrate for several enzymes known as NAD⁺ consumers (e.g. sirtuins and PARPs) that regulate key biological processes. Due to the emerging importance of cellular NAD⁺ metabolism, the goal of our research is to understand how NAD⁺ homeostasis is maintained. Our lab recently discovered that NAD⁺ insufficiency leads to delayed development of the gonad in *C. elegans*. Using Global Metabolomic Profiling, we discovered that our *pnc-1* mutants experienced glycolytic blockage where glyceraldehyde-3-phosphate (G3P) levels are up and 3-phosphoglycerate (3-PGA) levels are down. However, the level of pyruvate is inconsistent with the notable trends of G3P and 3-PGA. Restoring glycolysis, by feeding 3-PGA and phosphoenolpyruvic acid (PEP) metabolites, rescued this gonad delay phenotype in our *pnc-1* mutants. Although we noticed glycolytic blockage, we had evidence that pyruvate levels and mitochondrial functions were not changed in our *pnc-1* mutants indicating that the TCA cycle was not affected by NAD⁺ insufficiency. This led us to the question that if glucose is not being converted to pyruvate, then how are the levels and functions of pyruvate unchanged? Therefore, we hypothesized that excessive use of amino acids as an energy source compensates for insufficient glycolysis flux in *pnc-1* mutants. We performed targeted metabolomics and measured the levels of alanine and cysteine. Our results revealed that both alanine and cysteine show a 2-fold increase in our *pnc-1* mutants. We also observed an up-regulation of aminotransferase mRNA expression of important enzymes involved in amino acid catabolism. We are currently measuring the flux of isotope labeled glucose, alanine and cysteine to pyruvate in both N2 and *pnc-1* animals. If the turnover rate of alanine and/or cysteine is higher in our *pnc-1* mutants, then this data supports our hypothesis. Whereas, if the turnover rate of glucose is lower in our *pnc-1* animals, then this data supports the notion that glycolytic blockage is occurring due to NAD⁺ insufficiency. We interpret these data as evidence that excessive amino acid use as an energy source is compensating for glycolysis flux in *pnc-1* mutants.

P1537**Board Number: B1039****Hindsight regulates photoreceptor axon targeting through transcriptional control of jitterbug/Filamin and multiple genes involved in actin regulation and axon targeting in *Drosophila*.**

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The establishment of well-defined synaptic connections during development is a key step in the maturation of the nervous system of vertebrates and invertebrates. Previously, by using a microarray analysis, we have shown that the transcription factor Hindsight (HNT) controls the expression of a group of genes involved in axon guidance and actin regulation.

However, it remains unclear how HNT controls the expression of these genes and how they function to regulate axon targeting.

To tackle these questions, first we performed loss of function (LOF) analysis, which confirmed the role of *otk/PTK7* in photoreceptor axon targeting and uncovered Tiggrin, an integrin ligand, and *Jbug/Filamin*, a conserved actin-binding protein, as new factors that participate of photoreceptor axon targeting. Since HNT LOF results in diminished expression of *jbug/Filamin* and *otk* we hypothesized that HNT acts directly through them to regulate axon targeting. To test this idea, we overexpressed *jbug/Filamin* in a HNT mutant background to verify whether the phenotype is rescued. Our data indicates that HNT acts partially through *jbug/Filamin* to regulate axon targeting.

Finally, to confirm our previous bioinformatic data on the transcription regulation of *jbug/Filamin*, we performed a promoter analysis *in silico* and by using Chromatin Immunoprecipitation and EMSA. Our data showed that HNT binds directly to a new consensus motif, suggesting that HNT regulates directly and positively the expression of *jbug/Filamin*.

Our work sheds light into the understanding of how transcription factors coordinate the expression of a group of genes involved in actin regulation and axon targeting in order to achieve the correct connectivity pattern in the nervous system. Further studies will be required to clarify the mechanism by which these genes regulate axon targeting.

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P1538**Board Number: B1040****Blocking EGF signaling in perineurial glia during *Drosophila* metamorphosis leads to aberrant nerve branching patterns.**J.J. Fernandes¹, M. Siefert¹;¹Miami Univ, Oxford, OH

The pattern of larval abdominal nerves in *Drosophila* is reorganized during metamorphosis to generate a new adult pattern. This involves fusion of 5 sets of posterior abdominal nerves into a single terminal nerve trunk (TNT), which defasciculates in the body wall to innervate muscle targets. Each nerve is surrounded by three glial layers and this study aims to examine a role for the most external layer in bringing about nerve fusion. Using a layer specific Gal4 driver line, a dominant negative form of the EGF receptor (DN DER) was targeted to the perineurial layer, and was expressed during the 4 day period of metamorphosis. In the adults that emerged, the number of glial cells were examined using the anti-repo antibody, in addition to the pattern of nerves (visualized with anti- HRP). The number of repo-positive nuclei in experimental animals along the TNT just as it emerges from the abdominal ganglion was reduced by half (controls: 32.4 ± 3.2 ; experimental: 16 ± 0.8 ; $n=8$ for both groups; P

P1539**Board Number: B1041****Sac1 selectively regulates trafficking of adhesion molecules in the developing *Drosophila* eye.**L. Del Bel^{1,2}, R. Wilk¹, G. Polevoy², J. Burgess^{1,2}, J.A. Brill²;¹Molecular Genetics, University of Toronto, Toronto, ON, ²Cell Biology Program, Hospital for Sick Children, Toronto, ON

Phosphatidylinositol phosphates (PIPs) are membrane lipids that regulate many cellular events. They can act as signaling molecules, organelle identifiers and recruiters of effector proteins. Phosphatidylinositol (PI) 4-phosphate (PI4P) is the most abundant cellular PIP. PI4P is present on most membranes in the cell; however, it is enriched at the Golgi, where it recruits effectors of intracellular trafficking. PI4P-regulating enzymes have been well studied in yeast and cultured cells, yet little is known about their specific roles in animal development. In *Drosophila melanogaster*, three PI 4-kinases are responsible for generating PI4P, whereas a single phosphatase, Sac1, keeps PI4P levels in check. By generating *Drosophila* mutants, we discovered that proper regulation of PI4P by Sac1 is critical for eye development. Flies bearing a temperature-sensitive (ts) allele of *sac1* exhibit an eye pigmentation defect, as well as a rough eye phenotype due to defects in pigment cell morphogenesis.

During early pupal eye development (24-30hr after puparium formation, APF) we find that the Neph1 homolog Roughest, a transmembrane protein required for ommatidial patterning and pigment cell specification, is normally enriched at the plasma membrane. However, this enrichment is not observed

in *sac1^{ts}* mutants. As a result, *sac1^{ts}* mutants exhibit irregular, multi-layered interommatidial cells (pigment cell precursors), as well as disorganized bristles. Interestingly, Roughest's paralog Kirre, reaches the plasma membrane as expected, suggesting that Roughest regulation and trafficking alone is sensitive to alterations in PI4P. At later stages of pupal eye development (42hr APF), we find that interommatidial cells in *sac1^{ts}* mutants exhibit ER stress, as monitored by increased Xbp1 splicing. These cells also accumulate Myospheroid (β -integrin) and Notch, in addition to Roughest and Kirre. Our results demonstrate that proper regulation of PI4P by Sac1 is critical for trafficking of adhesion proteins and for maintaining ER homeostasis during *Drosophila* eye development.

P1540

Board Number: B1042

Regulatory Role of miR-124 in Early Development.

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microRNAs (miRNAs) play a crucial role in cell differentiation. The brain-specific miR-124 is highly conserved in both invertebrates and vertebrates. We propose to use the purple sea urchin, *Strongylocentrotus purpuratus*, to examine the function of miR-124. The Delta/Notch is a conserved signaling pathway that regulates neural differentiation and it also activates GCM that is critical for the specification of mesodermally-derived pigment cells. We bioinformatically identified Notch and GCM to contain potential miR-124 binding sites. We hypothesize that miR-124 regulates the Delta/Notch signaling pathway important for neurogenesis and mesodermal specification. Using luciferase reporter constructs and site-directed mutagenesis, we will test the direct regulation of miR-124 of Notch. Dose-dependent miR-124 loss-of-function induced phenotypes include a significantly larger midgut width, abnormal cell sizes, and cell clumps at the gastrula stage (48 hours post fertilization). This study elucidates the function of miR-124 by identifying its direct gene targets and contributes to our understanding of neural development and mesodermal specification in the early embryo.

P1541

Board Number: B1043

Microtubule-dependent apical restriction of recycling endosomes sustains adherens junctions during morphogenesis of the *Drosophila* tracheal system.

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Epithelium remodelling is an essential mechanism for organogenesis during which cells change shape and positions while maintaining contact with each other. Adherens junctions (AJs) mediate stable cohesion between cells but must be actively reorganised to allow morphogenesis. Vesicle trafficking and

the microtubule (MT) cytoskeleton contribute to regulating AJ plasticity but their cross relationship remains elusive. We carried out a detailed analysis of the role of MTs in cell remodelling during the formation of the embryonic respiratory organ of *Drosophila*. The so called tracheal system is a very well characterised multicellular organ composed of highly ramified epithelial branches. We induced MT depolymerisation specifically in tracheal cells, and found that MTs are essential during a specific time frame corresponding to tracheal cell elongation that drives branch outgrowth. In the absence of MTs, tracheal cells over-elongated ultimately leading to branch breaks. We established through 3D quantifications that MTs are critical to sustain E-Cadherin and Par-3 levels at AJs. Furthermore, maintaining E-Cadherin/Par-3 levels at the apical domain requires de novo synthesis rather than internalisation and recycling from and to the apical plasma membrane. However, we showed that the apical targeting of E-Cadherin and Par-3 requires the functional recycling endosome, suggesting an intermediate role for this compartment in sorting de novo synthesized E-Cadherin to the plasma membrane. Our results further demonstrated that, in tracheal cells, the observed apical enrichment of the recycling endosome is dependent on the MT motor Dynein, and essential for its function. We revealed that E-Cad distribution in tracheal cells, undergoing 3D remodelling through collective cell migration, is more sensitive to MT depolymerisation and to the function of the recycling endosome than the overlying 2D ectodermal cells. Altogether, our results uncover an MT-Dynein-dependent apical restriction of recycling endosomes that controls adhesion by sustaining Par-3 and E-Cadherin levels at AJs during organ formation.

P1542

Board Number: B1044

Twist signaling and intercellular coupling coordinate pulsed and ratcheted apical contractions during tissue folding.

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For multicellular tissues to change shape, dynamic cell shape changes must be coordinated across the tissue to give rise to the correct pattern of forces and geometries that will restructure tissue architecture. A ubiquitous mechanism responsible for cell shape change is actin-myosin contraction, in which the molecular motor myosin contracts cortical networks of filamentous actin (F-actin) to generate tensile force. In a wide variety of developmental contexts, actin-myosin contractions occur in discrete force generating events, called pulses, in which myosin cyclically contracts an F-actin meshwork. A well-studied example is apical constriction during *Drosophila* ventral furrow formation, in which collective apical constriction causes epithelial folding and cell invagination. In this system, cycles of rapid constriction are stabilized through a proposed ratchet mechanism that is dependent on high levels of the transcription factor Twist. To investigate how these two modes of contractility, pulse and ratchet, are spatiotemporally coordinated through signaling or intercellular coupling, we developed a computational approach that systematically identifies contractile events as well as makes quantitative

measurements of their location, timing, and ratchet engagement. We find that Twist signaling in ventral furrow cells promote the transition from unratcheted, weak, and lower-frequency pulses to ratcheted, stronger, and higher-frequency pulses. We also show that pulses are spatially coordinated; both cell-autonomous and non cell-autonomous factors influence ratchet engagement. Ratcheting is associated with persistent myosin structures within a cell as well as enrichment in having pulses co-occur in next-neighbor cells. In addition, neighboring ratcheted pulses act cooperatively to constrict the cells' apical areas. Our findings show that coordinated force generation arises from the interplay between a global signaling program and intercellular coupling between individual cells of the epithelium.

P1543

Board Number: B1045

Macroglobulin complement related (Mcr) and Thioester protein 3 (Tep3) demonstrate a requirement for thioester-containing proteins in *Drosophila* development.

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Thioester-containing proteins (TEPs) are conserved among vertebrates and invertebrates and include the complement and macroglobulin families of proteins. TEP proteins have been studied for their role in innate immunity and have been found to aid in the phagocytosis of pathogens. Our lab recently characterized one of the six *Drosophila* TEP genes, *Macroglobulin complement related (Mcr)*, as having an essential developmental role in the establishment and maintenance of the epithelial septate junction, the invertebrate barrier junction that is functionally analogous to the vertebrate tight junction. Interestingly, a recent examination of the vertebrate TEP gene, *CD109*, also suggested a developmental requirement, indicating that this protein family may have roles in essential developmental processes in addition to innate immunity. Our lab is investigating if this dual role is specific to *Mcr* and *CD109* or is a conserved feature of additional TEP genes. To examine this question, we conducted a lethal phase analysis and found that four of the six *Drosophila* TEP genes contribute to one or more developmental processes. *Thioester protein 3 (Tep3)* encodes a protein with a signal sequence, a potential integrin binding site, and a complete macroglobulin domain. Mutations in *Tep3* result in an extended lethal phase with a variety of terminal phenotypes, including persistent salivary glands. Autophagy dependent programmed cell death is required for the destruction of larval salivary glands during metamorphosis and requires the engulfment receptor Draper. The localization of Draper in salivary glands requires *Tep3*, suggesting a role for *Tep3* in developmental autophagy.

P1544**Board Number: B1046****ROCK organizes ratcheted apical constriction in a folding epithelium by continuously stabilizing apical myosin.**J.S. Coravos¹, A.C. Martin¹;¹Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA

Apical constriction transforms epithelial cells from a columnar to a wedge or cone shape, which can result in bending and folding of epithelial sheets during organogenesis. Apical constriction in many systems, including *Drosophila* gastrulation, occurs through pulses of rapid contraction, with reduced apical area being maintained between pulses. These dynamics suggest that apical constriction requires a ratchet-like component. A proposed mechanism for ratcheted constriction is that Rho-associated coiled-coil kinase (ROCK) not only elicits contractile pulses through dynamic myosin phosphorylation, but also subsequently stabilizes an apical actomyosin meshwork that restrains apical area against opposing stresses. This model is difficult to test because genetic inactivation of ROCK or early inhibitor injection prevents all apical myosin accumulation. Here, we present a novel approach in which we can image an embryo while simultaneously injecting it with an inhibitor. By giving us both precise temporal control of the perturbation, and observations of the immediate response, this approach allows us to probe the requirement of ROCK in stabilizing cell shape after constriction has initiated. We find that ROCK inhibition leads to immediate relaxation of apical area and loss of apical actin and myosin organization. This result suggests that continuous ROCK activity organizes the molecular ratchet through maintenance of actin and myosin in the apical domain of apically constricting cells.

P1545**Board Number: B1047****Regulation of adherens junction stability by intracellular pH dynamics.**B.K. Grillo-Hill¹, M. Esquivel¹, K.A. White¹, D.L. Barber¹;¹Cell and Tissue Biology, University of California, San Francisco, San Francisco, CA

Changes in intracellular pH (pHi) are linked to several developmental processes, however the role of pHi dynamics in cell differentiation and epithelial plasticity remains unexplored. We found that increased pHi is necessary for the differentiation program of epithelial-mesenchymal transition (EMT). With EMT of human lung alveolar and mouse mammary epithelial cells induced by TGF- β , we found that pHi increased ~ 0.3 units and we found increased activity of the plasma membrane Na⁺-H⁺ exchanger NHE1, a major regulator of pHi homeostasis. NHE1 shRNA specifically prevents increased pHi and blocks EMT, including dissolution of adherens junctions, actin stress fiber formation and transcriptional reprogramming. We also found that increasing pHi in the absence of TGF- β was sufficient to decrease β -catenin levels at adherens junctions. To test a role for β -catenin in pH-dependent dissolution of adherens junctions, we investigated genetic interactions in *Drosophila*. We found that retinal overexpression of *Dnhe2*, the ortholog of NHE1, increases pHi and disrupts cell architecture, manifested

as a “rough eye” phenotype. Overexpression of *armadillo* (*arm*, ortholog of β -catenin) suppresses this phenotype, while RNAi knockdown of *arm* enhances it. These data suggest that β -catenin may be a pH sensor with decreased stability at higher pH. β -catenin degradation is predominantly mediated by GSK3- β phosphorylation of Ser33 and Ser37, which promotes recognition and degradation by the ubiquitin-proteasome pathway. We predicted His36, located between Ser33/37 might function in pH sensing with a neutral His36 at higher pH decreasing stability and a protonated His36 at lower pH increasing stability. Although His36 is evolutionarily conserved, its functional significance has not been reported. However, the COSMIC database indicates a recurring β -catenin-H36R mutation in distinct cancers, which generally have increased pH_i. We predicted that a substitution with a positive charge would prevent pH-regulated β -catenin degradation. We confirmed this prediction by showing that overexpression of *armH47R* (analogous to human H36R) but not *armH47A* or *armWT* phenocopies the gain-of-function *armT52A* that is nonphosphorylatable and resistant to degradation. We are currently testing whether charged His36 at lower pH increases β -catenin stability by electrostatically interfering with Ser33/37 phosphorylation or E3 ubiquitin ligase binding. Our data indicate the new finding of pH sensing by β -catenin, with decreased stability at higher pH_i, and reveal a novel mechanism for remodeling of cell-cell junctions by dynamic changes in pH_i. Additionally, many cancers have increased pH_i, suggesting that oncogenic roles for β -catenin may also be regulated by pH_i.

P1546

Board Number: B1048

Cell Intercalation in *Drosophila* Germ-Band Extension Is Controlled Through Local Regulation of Junction Sliding.

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The elongation of the central body axis is a fundamental morphogenetic process in embryogenesis and organogenesis. One widely studied example of this process is germ-band extension (GBE) in *Drosophila*, where the germ-band epithelium elongates along the anterior-posterior (AP) axis while simultaneously narrowing in the dorsal-ventral (DV) direction. This process is driven by cell intercalation, where a junction between AP neighbors fully contracts to a common vertex, which in turn resolves directionally through the formation of a new junction between DV neighbors. In *Drosophila* GBE, the symmetry-breaking cues for intercalation are provided by a system of planar polarized localization of acto-myosin and adhesion proteins, which are concentrated at AP or DV junctions, respectively. Based on the requirement for myosin and on measurement of the line tension of these junctions in laser dissection experiments, the current mechanistic model holds that shortening of AP junctions is caused by an increase in AP line tension mediated by the contraction of apically enriched actomyosin superstructures. Physically, this line tension model predicts that (1) contraction of AP junctions should occur through mechanically coupled inward motion of junction vertices, and that (2) contraction should be initiated from the apical end. Using a Matlab-based suite for automated computational image analysis, we have tracked junction remodeling during GBE with high spatial and temporal resolution. We found no

evidence that vertex motion during GBE junction remodeling is mechanically coupled, but that vertices undergo independent and reversible step-like motion. In addition, instead of systematic propagation from the apical to the basal end, we found that remodeling events - including contractions of AP junctions - are initiated both apically and basolaterally. Based on these results, we suggest that junction remodeling is *not* directly driven by apical line tension anisotropy; rather, junction remodeling is the result of directional local sliding motion of individual vertices, which is driven by a pool of actomyosin that operates on the entire length of the apical-basal axis, and that regulates adhesion and/or membrane traffic events through a mechanism based on locally confined and anisotropic contraction.

P1547

Board Number: B1049

A maternal-zygotic module stabilizes F-actin to promote robust morphogenesis.

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Robustness is a property built into biological systems to ensure that the outcome of an event is reliable even when inputs, like gene dosage and environment, vary. During development, robustness safeguards embryos against mistakes that underlie structural and functional birth defects. Yet, our knowledge of how robustness is achieved during mechanical events like embryonic morphogenesis is very limited.

Here we show that the tissue-building event of *Drosophila* cellularization is made robust by mechanisms targeting F-actin. We find that F-actin levels are significantly reduced in cellularizing embryos upon high temperature perturbation. This F-actin reduction leads to failures in cellularization and embryos that do not hatch. Using a genetic strategy, we identified two novel members of the Vinculin/ α -Catenin Superfamily that work together to reinforce F-actin against such perturbations (Zheng et al., 2013). We find that zygotically-expressed *serendipity- α* (*sry- α*) and maternally-loaded *spitting image* (*spt*) are paralogs that share an overlapping, actin-stabilizing activity during cellularization. *spt* alone is sufficient for cellularization at optimal conditions, but both *spt* plus *sry- α* are required at high temperature and when the actin cytoskeleton is genetically compromised by a reduced dose of the actin accessory protein Profilin.

Strikingly, we find that *Sry- α* and *Spt* expression is precisely controlled, and that there is crosstalk between them. Specifically, *Spt* provides pre-cellularization functions that *Sry- α* cannot provide; and expressing elevated levels of *Spt* alone is not an option because it induces gain-of-function phenotypes. In addition, reducing maternal *spt* dosage triggers a compensatory up-regulation of zygotic *sry- α* transcription.

We suggest that *Sry- α* and *Spt* represent a robustness-promoting module for cellularization: Cross-talk between maternal *spt* and zygotic *sry- α* demonstrates that “paralog interactions” can span between maternal and zygotic genomes to promote robustness. In addition, we show for the first time that specific cytoskeletal-based mechanisms promote robust morphogenesis. Since all morphogenesis depends on cytoskeletal remodeling, in embryos and adults, robustness-promoting mechanisms aimed at F-actin could be effective at all life stages.

P1548

Board Number: B1050

Myosin II regulation and activity provide spatial and temporal control of the forces that shape tissues.

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During development, the forces generated by actomyosin contractility are controlled in space and time to shape simple epithelia into tissues with complex form and structure. Myosin II regulation and force generation have been extensively studied *in vitro*, but how these molecular properties are translated to longer lengthscales to achieve the patterns of forces that shape tissues is poorly understood. In the *Drosophila* embryo, planar polarized patterns of myosin activity promote oriented cell rearrangements that rapidly elongate the body axis from head to tail. To gain insight into how tissue elongation arises from molecular-level myosin activity, we generated a collection of transgenic lines expressing myosin II variants with altered molecular properties, including variants that disrupt regulatory phosphorylation or alter the speed at which myosin translocates actin filaments. We are using quantitative live imaging, FRAP, and laser ablation to analyze myosin dynamics and activity during tissue elongation. We find that both classes of myosin variants disrupt elongation, but have distinct effects at the molecular and cellular levels. First, we manipulated myosin regulatory light chain (RLC) phosphorylation, which promotes the assembly of inactive myosin monomers into active contractile filaments. These phosphovariants reveal that regulated myosin phosphorylation influences both the level and the spatiotemporal pattern of myosin activity. In embryos expressing phosphomimetic, constitutively active RLC, myosin is more stably associated with the cell cortex and cells rearrange faster and participate in more higher-order collective rearrangements. In embryos expressing unphosphorylatable, inactive RLC, myosin turns over faster at the cortex and there are fewer, slower cell rearrangements. Strikingly, myosin planar polarity and mechanical anisotropy are reduced in embryos expressing either phosphovariant, associated with poorly oriented cell rearrangements and less efficient tissue elongation, indicating that regulated myosin phosphorylation is essential for controlling the spatiotemporal patterns of myosin activity in this tissue. In addition, we manipulated the myosin motor domain to produce variants predicted to alter the speed at which myosin translocates actin filaments. Like constitutively active myosin, motor variants predicted to slow myosin translocation are more stably associated with the cortex, but produce distinct myosin localization patterns, cell behaviors, and tissue elongation phenotypes. These quantitative live imaging

studies provide insight into how specific features of myosin activity at the molecular level give rise to the spatially and temporally regulated forces that shape tissues.

P1549

Board Number: B1051

Tight apposition and multiple cell-cell contacts mediate myoblast fusion in *Drosophila*.

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Myoblast fusion is an essential process underlying development, growth and regeneration of muscles in multicellular organisms. Our work focuses on an ultra-structural description of the fusion process during formation of the indirect flight muscles (IFMs) of *Drosophila*. These large muscles, which display a variety of structural features analogous to those of vertebrate muscles, are generated through fusion of numerous myoblasts with prominent myotube templates, making them an attractive subject for study of myoblast fusion in an experimental system amenable to genetic investigation. In order to study fusion between IFM myotubes and myoblasts, we have employed a novel tissue preparation protocol, which allows for high-quality preservation and visualization of cell membranes, and a variety of transmission electron-microscopy (TEM) techniques (FIB/SEM, single section TEM and STEM tomography). Using these methods, we have identified a series of distinct steps in the IFM myoblast fusion process, mediated, as genetic analysis suggests, by different cellular machineries. Cell-adhesion elements are essential for an initial association between the myoblasts and myotubes, bringing them in close (~25nm) proximity to each other. Myoblasts then flatten their surface and move even closer (~10nm) to the myotubes, a transition that is associated with formation of multiple point-like contact sites between the two cell types. Components of the Arp2/3 branched-actin polymerization machinery, established mediators of myoblast fusion in *Drosophila*, as well as the MARVEL-domain transmembrane protein Singlet-bar, are required for these events. The myotube-myoblast contact sites, which form along long stretches of the apposed membranes, and are not associated with membrane protrusions, give rise to multiple nascent fusion pores, which perforate the cell membranes. The pores grow in size, leading to cell membrane breakdown and completion of the fusion process.

P1550**Board Number: B1052****Mechanical forces drive vertex resolution during *Drosophila* axis elongation.**J. Yu¹, R. Fernandez-Gonzalez^{1,2,3};¹Institute of Biomaterials and Biomedical Engineering, University of Toronto, Toronto, ON, ²Department of Cell and Systems Biology, University of Toronto, Toronto, ON, ³Developmental and Stem Cell Biology Program, The Hospital for Sick Children, Toronto, ON

Convergent extension, a conserved process in which a tissue elongates along one axis and narrows along a second orthogonal axis, is necessary for kidney tubule, limb bud, and neural tube elongation. In the *Drosophila* embryo, the elongation of the anterior-posterior body axis is driven by convergent extension largely mediated by cell intercalation. During intercalation, cell interfaces parallel to the dorsal-ventral axis of the animal contract, forming transient vertices where four or more cells meet. These multicellular vertices are resolved through the assembly of new cell interfaces parallel to the anterior-posterior axis of the animal. Currently, the mechanisms behind new interface assembly during vertex resolution are poorly understood.

Vertex resolution is accompanied by the accumulation of actin at the resolving interface, and mechanical tension can promote actin polymerization. To determine if new cell interfaces during axis elongation sustain mechanical tension, we used laser ablation to sever newly-forming interfaces. The velocity of retraction of the tricellular vertices on either side of the ablation site is proportional to the tension sustained by the severed interface. Using particle tracking velocimetry to quantify recoil velocity, we found that new cell interfaces resulting from vertex resolution sustained 32% greater mechanical tension than older, more stable interfaces with a similar orientation. To determine if mechanical tension is sufficient to increase the rate of new interface assembly, we applied ectopic tension parallel to the direction of interface resolution using laser nanosurgery, and we found that the elongation rate of new interfaces increased 1.81-fold when compared to control embryos. Together, these data suggest that mechanical forces oriented along the anterior-posterior axis of the animal drive vertex resolution during axis elongation in *Drosophila*. We previously showed that intercalating cells undergo pulsatile contractions of their apical surfaces, and that the amplitude of these contractions is greater along the anterior-posterior axis of the embryo. Using quantitative image analysis, we have found that new interfaces resulting from vertex resolution do not elongate in a continuous manner; rather, elongation occurs in pulses. We are currently investigating whether pulses of new edge elongation are correlated with pulses of apical constriction in the cells anterior and posterior to the new interface. Characterizing the mechanical signals behind axis elongation will be crucial in understanding the cellular and molecular mechanisms of animal development, as well as the causes of birth defects such as spina bifida and limb malformations.

P1551**Board Number: B1053****Compensatory branching morphogenesis of stalk cells in the *Drosophila* trachea.**D. Francis¹, A. Ghabrial¹;¹Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA

Tubes are essential for nutrient transport and gas exchange in multicellular eukaryotes, but how connections between different tube types are maintained over time is unknown. In the *Drosophila* tracheal system, mutations in oak gall (*okg*) and conjoined (*cnj*) confer identical defects, including late onset blockage near the terminal cell (TC)-stalk cell (SC) junction, and ectopic extension of autocellular, seamed tubes into the TC. We determined that *okg* and *cnj* encode the E and G subunits of the vacuolar ATPase (vATPase), and showed that both V0 and V1 domains are required for TC morphogenesis. Remarkably, the ectopic seamed tubes running along vATPase-deficient TCs belonged to the neighboring SCs. All vATPase-deficient tracheal cells had reduced apical domains, and TCs displayed mislocalized apical proteins. Further, TCs mutant for apical polarity genes, *par6* or *aPKC*, had ectopic seamed tubes. We thus identify a novel mechanism of compensatory branching in which SCs extend autocellular tubes into neighboring TCs with undersized apical domains.

Stem Cells and Pluripotency**P1552****Board Number: B1054****Molecular ties between the cell cycle and differentiation in embryonic stem cells.**V. Li¹, M.W. Kirschner^{1,2};¹Department of Systems Biology, Harvard Medical School, Boston, MA, ²Systems Biology, Harvard Medical School, Boston, MA

Attainment of the differentiated state during the final stages of somatic cell differentiation is closely tied to cell cycle progression. Much less is known about the role of the cell cycle at very early stages of embryonic development. Here we show that molecular pathways involving the cell cycle can be engineered to strongly affect embryonic stem cell differentiation at early stages in vitro. Strategies based on perturbing these pathways can shorten the rate and simplify the lineage path of ES differentiation. These results make it likely that pathways involving cell proliferation intersect at various points with pathways that regulate cell lineages in embryos and demonstrate that this knowledge can be used profitably to guide the path and effectiveness of cell differentiation of pluripotent cells.

P1553**Board Number: B1055****The influence of glucose concentration on the pluripotency of mouse embryonic stem cells.**C. Yoneda¹, K. Hayakawa¹, M. Hirosawa¹, S. . Tanaka¹, K. Shiota¹;¹Dep. of Anim. Res. Sci., The University of Tokyo, Tokyo, Japan

Cells can sense their nutritional status and adapt to various circumstances. Information regarding the glucose concentration can be transmitted to the nucleus through the O-GlcNAcylation of various molecules, which is catalyzed by O-linked N-acetylglucosamine transferase (OGT). Pluripotent cells including ES cells and iPS cells were established and maintained in a medium with relatively high glucose content (25 mM), whereas other cell types were cultured in lower glucose concentrations (~5 mM). To understand why the pluripotent stem cells favor hyperglycemic conditions, we studied the influence of low and high glucose concentrations on the growth, differentiation and expression of pluripotency-related genes and epigenetic regulators in mouse ES cells (mESCs). The mESCs cultured in 1, 5, and 25 mM glucose did not show a significant difference in their proliferation. Analysis of the gene-expression profiles of pluripotency markers revealed that the culture with 1 mM and 5 mM glucose for 4 days showed decreased *Dppa3* expression and unaltered expression of *Nanog*, *Klf4*, *Oct4*, *Sall4*, *Dppa2*, *Dppa4*, and *Zfp42*. This suggested that mESCs could harbor a set of glucose-sensitive genes. Considering the fact that *Dppa3* is involved in pluripotency and that it is the master gene for germ cell differentiation, we investigated the effects of low glucose concentrations on the pluripotency of mESCs by studying embryoid body (EB) formation. The shape and size of EBs were similar among the mESCs pre-cultured with low and high levels of glucose for 4 days. Intriguingly, the expression of the marker genes for the endoderm (*Gata4*, *Gata6*), mesoderm (*Kdr*), ectoderm (*Nog*) and germ cells (*Dppa3* and *Blimp1*) was reduced in mESCs pre-cultured in a low-glucose medium. We conclude that cells sense the glucose concentration in the surrounding environment and alter the expression of a specific set of genes that are involved in pluripotency or differentiation. We analyzed the expression of epigenetic factors to explore the mechanism of glucose concentration-dependent regulation of gene expression in mESCs and found an increase in OGT expression and O-GlcNAcylation in mESCs cultured in a low-glucose medium. Furthermore, we also observed an increase in the expression of *Ezh2* and *Suz12*, the components of the polycomb repressive complex 2, which catalyzes the trimethylation of lysine 27 of histone H3. In summary, mESCs require hyperglycemic conditions to maintain pluripotency. The glucose concentration sensed during the undifferentiated state is retained as a "glucose memory" that affects the differentiation capacity of mESCs, through alterations in the expression of epigenetic factors and the process of O-GlcNAcylation.

P1554**Board Number: B1056****Zebrafish intestinal stem cells are located at the base of the inter-villus pocket between villi ridges and populate both sides of the flanking ridges.**S. Tavakoli¹, P.T. Matsudaira¹;¹Mechanobiology Institute, National University of Singapore, Singapore, Singapore

In contrast to the stereotypic villus-crypt organization of the bird and mammalian intestine, the zebrafish intestinal epithelium is folded into villar ridges and crypts are absent. Previous studies have shown the dividing cells are located at the base of the villus ridge while cell death occurs at or near the villus tip. This simple organization suggests that intestinal stem cells (ISCs) maintain the villus epithelium along a base-to-tip axis. We tested the dynamics of zebrafish intestinal epithelium renewal by label retention assay, creation of mosaic intestinal tissues and finally lineage tracing. We examined the intestinal stem mammals' cell markers candidate in zebrafish. The promoters of *prmt1* and *Irig1* genes, as the most reliable intestinal stem cell markers in zebrafish, were used to express CreER^{T2} in the generated transgenic lines. Activating the CreER^{T2} at desired time helps to trace the Cre mediated recombination in tissue. Our result suggests that renewal of the zebrafish epithelium is similar to birds and mice with the newly divided cells at the base of the inter-villus pocket, which complete their translocation to the ridge. Furthermore, similar recombination pattern of the inter-villus pocket flanking sides of the villi ridges, demonstrates the bilateral migration of newly reproduced cells toward the flanking ridges. In another words, intestinal stem cells are located at the inter-villus pocket between villi ridges and populate facing ridges. Therefore, the facing ridges share the stem cells at the base and show similar expression pattern. In contrast, two sides of a ridge may show different expression pattern as originate from different stem cells.

P1555**Board Number: B1057****The neurofilament-derived peptide NFL-TBS.40-63 targets neural stem cells but does not affect their microtubule network.**J. Eyer¹, C. Lepinoux-Chambaud²;¹INSERM, Angers, France, ²Université d'Angers, Angers, France

Neural stem cells (NSC) are characterized by their capacity to self-renew, form neurospheres in culture, proliferate, and generate neurons, astrocytes and oligodendrocytes (multipotency). These characteristics provide new therapeutic strategies for the treatment of neurodegenerative disorders or malignant glioma.

We previously showed that a peptide corresponding to the tubulin-binding sequence located on the neurofilament light chain (NFL-TBS.40-63), alone or linked to nanoparticles, is able to target glioblastoma cells in vitro and in vivo. The selective and massive uptake of this peptide by glioblastoma

cells occurs through endocytic pathways and is related to their high proliferative state, whereas a low level of internalization occurs for slow proliferative healthy cells (astrocytes, oligodendrocytes or neurons). Moreover, while the peptide is able to disrupt the microtubule network of glioblastoma cells and inhibit their proliferation, it has no major effect on the microtubule network from healthy cells. Finally, when injected in rats bearing glioblastoma, the peptide reduces tumour development (Bocquet et al., 2009 J. Neurosci. 29, 11043-11054; Berges et al., 2012 Mol. Ther. 20, 1367-1377; Balzeau et al., 2013 Biomaterials 34, 3381-3389; Lepinoux-Chambaud & Eyer, 2013 Int J Pharm. 454, 738-747).

Here, we show that this peptide is able to translocate passively in neural stem cells in vitro and in vivo. The microtubules of these cells show no detectable alteration by the peptide. Moreover, the in vitro formation of neurospheres and the cell viability are not affected by the peptide. However, the NSC capacity to self-renew is reduced with the peptide and is associated with an increase of adherent cells and a decrease of NSC proliferation. Finally, when injected in the cerebrospinal fluid of rats the peptide targets adult neural stem cells in vivo without major detectable cytotoxicity. These results indicate that this peptide represents a new molecular tool to target neural stem cells in order to develop new strategies for regenerative medicine and treatment of brain tumors. This peptide also allows the manipulation of the cytoskeleton of these neural stem cells and to investigate the consequences of such manipulations.

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P1556

Board Number: B1058

The Wnt signaling and cytoskeletal regulator APC2 controls stem cell niche size and architecture.

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Stem cell behavior is controlled by the cellular neighborhood, or niche, in which stem cells reside. Niche function relies on specific tissue architecture and cell-cell signaling to maintain tissue homeostasis. Signaling within the niche is well studied. However, regulation of niche architecture is not well understood. Here we report that the Wnt signaling and cytoskeletal regulator, Adenomatous polyposis coli (APC2) is required for proper niche size and architecture in the germarium. 5-7 Cap cells (Cpc), a central component of the female germline stem cell niche, reside at the anterior tip of the germarium and are essential for maintaining stem cells in the niche. Loss of APC2 results in an increased number of Cpc, and their displacement away from the anterior tip. Interestingly, there does not appear to be a direct relationship between the increase in Cpc number and the defect in Cpc position. In addition, APC2 null mutants exhibit an increased number of germline stem cells (GSCs) that may result from increased Cpc number. Preliminary data using a separation of function allele of APC2 suggest that Cpc number is

regulated by Wnt signaling, while Cpc position requires the actin regulation function of APC2. In the syncytial embryo, APC2 regulates cortical actin through a collaboration with the formin Diaphanous. We are currently testing the hypothesis that this collaboration regulates Cpc position. Consistent with our hypothesis, selective reduction of the actin organizers Spectrin and Kelch in Cpcs results in Cpc displacement, and Spectrin is mislocalized in APC2 mutants. These results suggest that the assembly and organization of cortical actin is required for proper Cpc position. Taken together, our data suggest that APC2 plays a dual role in the stem cell niche. APC2 regulates niche size by restricting Cpc number through negative regulation of Wnt signaling, and controls Cpc position within the germarium through the regulation of cortical actin.

P1557

Board Number: B1059

Lack of arginylation causes stem cells' inability to maintain pluripotency, leading to abnormalities during embryogenesis.

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Mice lacking arginyltransferase Ate1 die during embryogenesis with severe malformations in the heart, including thin myocardium, septation and outflow tract defects. The mechanisms of these malformations have been unknown and cannot be recapitulated in any conditional model with Ate1 knockout in heart-forming cell lineages, suggesting that these defects may originate prior to lineage specification. To investigate this possibility we have first attempted to generate Ate1 knockout stem cells, but failed to do so on multiple attempts, suggesting that these cells may have viability issues in culture or other reasons that preclude them from being generated. We next used primary embryonic fibroblasts derived from E12.5 wild type and Ate1 knockout embryos to generate induced pluripotent stem (iPS) cells by transfecting them with an expression cassette containing combination of reprogramming factors known to induce pluripotency (Oct4, Sox2, and Klf4). This approach was successful in yielding multiple Ate1 knockout iPS lines. These lines could maintain pluripotency in culture, however they showed a decrease in the intracellular level of several other pluripotent markers compared to the control. Moreover, removal of the expression cassette resulted in their inability to maintain pluripotency and induced their spontaneous differentiation into cardiomyocytes over the course of several days. Chimeras generated from these iPS cells died in development with prominent malformations and morphogenic defects. These data suggest that Ate1 is required to maintain normal intracellular levels of pluripotency factors in embryonic stem cells during early embryogenesis. We propose that cardiovascular malformations seen in Ate1 knockout embryos result from premature differentiation of cardiac progenitors into non-proliferating cardiomyocytes, which can occur before the progenitors can expand sufficiently to ensure normal cell numbers in the developing myocardium and

septae. This effect is likely universal and may affect other tissues and organs that do not have a chance to develop due to the timing of Ate1 knockout embryonic lethality.

P1558

Board Number: B1060

Limbal epithelial side population cells have an endothelial and/or mesenchymal-like phenotype with somatic stem or progenitor cell markers.

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Corneal epithelial stem cells are essential for maintaining homeostasis at the ocular surface. However, conclusive markers for corneal epithelial stem cells have not yet been defined. Our previous studies have demonstrated that rabbit limbal epithelial side population (rLE-SP) cells exhibit stem cell-like phenotypes including the enhanced expression of somatic stem cell markers that were commonly observed in other tissues. However, the unestablished database of rabbit genome sequences and the lack of available antibodies for rabbit antigens have made further characterization of rLE-SP cells difficult. Here, we show the gene expression profiles of both rLE-SP and rLE-non SP cells through RNA sequencing in which reads are mapped to both rabbit-derived sequences in the public database and the contigs that were created by de novo assembly. Firstly, we compared gene expression levels between rLE-SP and rLE-non SP cells, and confirmed that this gene expression analysis enables to recapitulate our previous results: rLE-SP cells showed higher gene expression of stem cell markers such as ATP-binding cassette sub-family G (*Abcg2*), polycomb complex protein BMI-1 (*Bmi1*), and *Nestin* than rLE-non SP cells. In addition, genes that were upregulated more than 5-fold in rLE-SP cells were identified using RNA sequencing, and five of these genes were selected for further analysis: ATP-binding cassette sub-family B (*Abcb1*), *vascular cell adhesion protein1 (Vcam1)*, Vimentin (*Vim*), *CD34*, and *Kit*. The differential expression of all of these genes was validated using real-time polymerase chain reaction. Expression of *Abcb1* has been detected in embryonic stem cells, induced pluripotent stem cells, and cancer stem cells. In addition, *CD34*, *Vcam1*, and *Kit* are known as somatic stem or progenitor cell markers. These findings support the hypothesis that immature cells are enriched in the rLE-SP cell fraction. On the other hand, expression of *Vim* has also been detected in endothelial, fibroblast, muscle, and mesenchymal cells. In addition, both *Vcam1* and *CD34* are also expressed in activated endothelial cells as well as mesenchymal stem cells. These findings indicate that rLE-SP cells have an endothelial and/or mesenchymal cell-like phenotypes rather than epithelial cells. In the present study, we have established a comprehensive gene expression analysis of rLE-SP cells using RNA sequencing, and the results suggest that rLE-SP cells have stem cell-like phenotypes. Specifically, we found that rLE-SP cells have an endothelial and/or mesenchymal cell-like phenotypes, which may imply a novel character and/or population of corneal epithelial stem cells.

P1559**Board Number: B1061****Analysis of gene expression in developing C2C12: changes in the proteome, titin and galectin-1.**S. Rashid¹, L. Gallagher², R. Giles¹, G.R. Walker¹;¹Biological Sciences, Youngstown State Univ, Youngstown, OH, ²School of Medicine, Trinity College Dublin, Dublin, Ireland

Myogenesis is a tightly regulated process resulting in unique structures called myotubes or myofibers, which compose skeletal muscle. Myotubes are multi-nucleated fibers containing a functional unit composed of cytoskeletal proteins called the sarcomere. The specific arrangement of these proteins in the sarcomere works to contract and relax muscles; literature suggests these proteins may even serve more than just a structural role but act as a scaffold for cell signaling, titin being of particular interest. During embryonic and post-embryonic development, fluctuations in expression of growth factors throughout the program account for the dramatic structural changes from cell to mature muscle fiber. In order to assess the dynamics of protein expression throughout myogenesis, we conducted a time course study using the mouse myoblast cell line C2C12, in which cells were allowed to differentiate, and insoluble protein fractions were collected at seven time points. The insoluble protein fraction accounts for cytoskeletal proteins, involved in the dramatic remodeling of cells. In order to discover influence of growth factors on protein expression, a similar time course was conducted with the of adding back to the cultures of growth factor (serum) at specific time points after induction of myogenesis. The cells were allowed to differentiate through the time course and the insoluble-protein fractions were collected. Analysis of protein expression during myogenesis shows significant changes in the insoluble proteome, Quantitative PCR shows an increase in titin expression and a transitory increase in galectin-1 expression.

P1560**Board Number: B1062****The Effects of Allogeneic Platelet Releasate Preparations on the Growth and Gene Expression of Human Adipose Derived Stem Cells.**M. McLaughlin¹, P. Gagnet¹, R. Yeager¹, S. Kapp¹, E. Cunningham¹, K. Guski¹, D. Kuebler¹;¹Biology, Franciscan University, Steubenville, OH

The administration of human adipose derived stem cells (ASC) represents a promising regenerative based therapy for joint injuries given the ability of these cells to undergo chondrogenic differentiation *in vitro*. These cells can be easily isolated from liposuction-derived adipose tissue and cultured under a variety of conditions to produce sufficient numbers of cells for treatment. Because different culture conditions are likely to have an effect on the physiology and therapeutic potential of these cells, we examined the effect of culturing ASCs in media supplemented with 10% allogeneic platelet releasate

(PR) as compared to standard 10% FBS supplemented media. PR was prepared from four patients using the Harvest SmartPrep® system followed by thrombin activation of the platelets. In every case, ASCs grown in media supplemented with PR proliferated much faster both during passage 0 (P0) and passage 1 (P1). The P1 cells in PR had an average doubling time of 27.1 ± 1.3 hours as opposed to 48.7 ± 5.7 hours in 10% FBS. In addition, the PR supplemented cells were smaller and more spindly in shape. The cells grown in PR retained the ability to differentiate along osteogenic, adipogenic and chondrogenic lineages and they retained expression of mesenchymal stem cell surface markers CD73 and CD90. In addition to assessing growth and differentiation potential, we also examined the effect that culturing the cells in 10% PR had on gene expression as compared to 10% FBS. We found that PR exposed ASCs displayed elevated expression of BMP-4 (5.7 ± 0.97 fold increase) and BMP-2 (4.7 ± 1.3 fold increase), both of which are implicated in cartilage development. The gene expression patterns of TGF-beta and VEGF were not significantly changed by PR exposure while decreased levels of gene expression were found in the cases of PDGF-B (4.0 ± 1.4 fold decrease) and FGF-2 (33 ± 9.0 fold decrease), two growth factors that are found at high levels in PR. These changes in gene expression were consistent across all four PR samples. In conclusion, the use of allogeneic PR to culture ASCs consistently leads to both an increased cell yield and a defined gene expression profile that may be beneficial for the treatment of joint injuries.

P1561

Board Number: B1063

Mouse Parthenote Stem Cell Genome Editing Using CRISPR/CAS9.

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Mouse parthenote stem cells are derived from unfertilized, activated mouse eggs, that can be either diploid or haploid. Genome editing at the time of activation therefore has the theoretical potential to give rise to uniformly modified chromosomes, leading to stem cell lines fully enriched for the gene modification of interest.

To test this possibility, we have designed a CRISPR/Cas9/signal RNA strategy to inactivate the CCR5 gene, an important co-receptor for HIV infection. B6C3 f1 mouse oocytes at the zygote stage were activated in a calcium/protein free medium supplemented with Strontium chloride and cytochalasin then micro-injected with an RNA transcript encoding Cas9 plus a signal RNA targeting a naturally occurring deletion in CCR5 known to inactivate CCR5 protein. Two of 19 micro-injected zygotes developed to the blastocyst stage, with one stable stem cell line derived from the inner cell mass of a hatched blastocyst cultured with standard stem cell culture protocol. At approximately 100,000 cells, half were analyzed for CCR5 gene mutations by sub-cloning and sequencing PCR products.

Twenty five percent of the clones exhibited CCR5 deletions at the sgRNA locus, indicating that one of 4 chromosomes was genome edited either at the late zygote or early two-cell stage. Interestingly, a DNA

construct of CCR5 sequences flanking a GFP reporter gene was not incorporated into the CCR5 region targeted by the sgRNA, but an homologous region of CCR2 was incorporated.

That 5% of microinjected parthenote zygotes gave rise to a stable cell line (now at passage 5) is an encouraging result. The 25% mutated DNA suggests either one blastomere at the four cell stage was homozygous for the CCR5 deletion, or two blastomeres at the four cell stage were heterozygous for the CCR5 deletion. Studies are in progress to distinguish between these two possibilities.

These results support the hypothesis that CRISPR/CAS genome editing of parthenotes at the haploid zygote stage may be a highly efficient system for deriving uniformly genetically modified stem cell lines, and significantly facilitate biological studies of early embryo development.

P1562

Board Number: B1064

c-MET Regulates Myoblast Motility and Myocyte Fusion during Adult Skeletal Muscle Regeneration.

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Adult muscle stem cells, satellite cells (SCs), endow skeletal muscle with tremendous regenerative capacity. Upon injury, SCs activate, proliferate, and migrate as myoblasts to the injury site where they become myocytes that fuse to form new muscle. How migration is regulated, though, remains largely unknown. Additionally, how migration and fusion, which both require dynamic rearrangement of the cytoskeleton, might be related is not well understood. c-MET, a receptor tyrosine kinase, is required for myogenic precursor cell migration into the limb for muscle development during embryogenesis. Using a genetic system to eliminate c-MET function specifically in adult mouse SCs, we found that c-MET was required for muscle regeneration in response to acute muscle injury. c-MET mutant myoblasts were defective in lamellipodia formation, had shorter ranges of migration, and migrated slower compared to control myoblasts. Surprisingly, c-MET was also required for efficient myocyte fusion, implicating c-MET in dual functions of regulating myoblast migration and myocyte fusion.

Host–Pathogen/Host–Commensal Interactions

P1563

Board Number: B1066

Role of Hazara virus infection in aquaporin expression and cell migration.

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Crimean-Congo hemorrhagic fever virus (CCHFV) is a human lethal pathogen which belongs to the *Bunyaviridae* family and causes Crimean-Congo hemorrhagic fever (CCHF), a severe disease characterized by hemorrhages, fever, muscle pain and other serious symptoms, and high mortality rates. CCHF may be transmitted to humans by the bite of ticks of the *Hyalomma* genus, which are present in many areas of Asia, Africa, Europe and the Middle East, and also by contact with blood or body fluids of infected livestock or humans. To date, there are some studies focusing on CCHFV-host cell interaction, the mechanisms of entry and release from cells and the replication cycle, but little is known about CCHFV effects on expression of aquaporins (AQPs) and cell migration.

Our aim was therefore to investigate how virus replication affects expression and distribution of AQPs and cell migration. Specifically, the role of AQP6 in cell migration is unknown, and it is further distinguished from other AQPs by exhibiting a strictly intracellular localization. Since the handling of CCHFV requires high containment biosafety level 4 laboratory facilities, we used Hazara virus as a model to study CCHFV. Using the wound healing assay, we investigated the effects of the virus on the migration of human epithelial intestinal Caco-2 cells. Moreover, we explored the effects of the virus on the expression of AQPs, through transfection of C3H10T1/2 mouse embryonic fibroblasts with AQP6.

We demonstrated for the first time that Hazara virus can inhibit epithelial sheet migration of Caco-2 cells and that the effects depend on the time of the infection and the virus concentration. Furthermore, we observed that AQP6 expression was reduced and cellular distribution was altered upon infection with Hazara virus. Finally, we developed a cellular assay that allows dynamic visualization of AQP6 in live cells over time and we propose a model of how virus infections affect epithelial sheet migration.

P1564**Board Number: B1067****Phosphorylation of HPV-16 E2 at serine 243 enables binding to Brd4 and mitotic chromosomes.**S. Chen¹;¹National Taiwan University, Taipei, Taiwan

The replication cycle of papillomaviruses is coupled to the differentiation program of the infected host keratinocytes. Infection is initiated when viruses enter the basal epithelial cells, where the viral genomes replicate to a low copy number and are maintained as extra-chromosomal episomes that replicate in synchrony with the host DNA. The papillomavirus E2 protein is involved in the maintenance of persistent infection and known to bind either to cellular factors or directly to mitotic chromosomes in order to partition the viral genome into the daughter cells. However, how the HPV-16 E2 protein acts to facilitate partitioning of the viral genome remains unclear. In this study, we found that serine 243 of HPV-16 E2, located in the hinge region, is crucial for chromosome binding during mitosis. Bromodomain protein 4 (Brd4) has been identified as a cellular binding target through which the E2 protein of bovine papillomavirus type 1 (BPV-1) tethers the viral genome to mitotic chromosomes. Mutation analysis showed that, when the residue serine 243 was substituted by glutamic acid or aspartic acid, whose negative charges mimic the effect of constitutive phosphorylation, the protein still can interact with Brd4 and colocalize with Brd4 in condensed metaphase and anaphase chromosomes. However, substitution by the polar uncharged residues asparagine or glutamine abrogated Brd4 and mitotic chromosome binding. Moreover, following treatment with the inhibitor JQ1 to release Brd4 from the chromosomes, Brd4 and E2 formed punctate foci separate from the chromosomes, further supporting the hypothesis that the association of the HPV-16 E2 protein with the chromosomes is Brd4-dependent. In addition, the S243A E2 protein has a shorter half-life than the wild type, indicating that phosphorylation of the HPV-16 E2 protein at serine 243 also increases its half-life. Thus, phosphorylation of serine 243 in the hinge region of HPV-16 E2 is essential for interaction with Brd4 and required for host chromosome binding.

P1565**Board Number: B1068****Dissecting the role of parafusin-related protein1 and calcineurin in Ca²⁺ signaling of *Toxoplasma gondii*.**S. Saha¹, B. Coleman¹, C. Chen¹, M. Gubbels¹;¹Biology, Boston College, Chestnut Hill, MA

Like any eukaryotic organism, Ca²⁺ signaling has been demonstrated to play pivotal roles in different cellular processes of the obligate intracellular apicomplexan parasite *Toxoplasma gondii*. In recent years several players of the Ca²⁺ signaling pathway have been mapped with critical roles in host cell invasion

and egress. Here we extend on this work by evaluating the functions of a putative phosphoglucomutase, parafusin-related protein 1 (PRP1), and the widely conserved Ca^{2+} dependent protein phosphatase calcineurin (CaN), which both have been associated with Ca^{2+} -dependent exocytosis (microneme secretion). Using fluorescent protein tagged constructs, we demonstrated the sub-cellular localization of the two proteins in live parasites. PRP1 localizes at the rhoptries (a Ca^{2+} independent secretory organelle) and inner membrane complex (IMC; cortical membrane cytoskeleton) in intracellular parasites while losing the IMC association in extracellular forms. CaN has cytoplasmic presence in intracellular parasites while in extracellular ones it colocalizes at both the apical and basal end with calmodulin. Direct knockout of PRP1 shows that the protein is non-essential. However, CaN is essential and its absence drastically reduces host cell attachment, but surprisingly microneme secretion is not affected. In conclusion, the roles of PRP1 and CaN are different than anticipated. Our current work aims to further our understanding of the exact role of CaN and PRP1 in *Toxoplasma* and where they fit in the overall Ca^{2+} signaling pathway in this highly successful group of pathogens.

P1566

Board Number: B1069

Effects of Quorum Sensing Signaling of *Pseudomonas aeruginosa* on Water Homeostasis in Human Primary Macrophages.

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The opportunistic Gram negative bacteria *Pseudomonas aeruginosa* cause chronic urinary tract, pulmonary and wound infections. They utilize the small molecule-based communication system called quorum sensing (QS) to regulate transcription of virulence genes and biofilm formation. The aim of the present study was to assess if QS signaling affects the cellular ability to phagocytose and the specific role of water homeostasis in this regard. The N-3-oxo-dodecanoyl-homoserine lactone (3O-C₁₂-HSL) and N-buturyl-homoserine lactone (C₄-HSL) are two important QS molecules that also affect human cells. Studying the effects of these QS molecules on human blood-derived macrophages we observed a stimulatory effect on phagocytosis of *P. aeruginosa* PAO1 wt compared to the QS-mutant lasI⁻/rhlI⁻ strain. During phagocytosis the plasma membrane needs to be highly dynamic to allow changes in cell shape and migration. Aquaporins (AQPs) are water channels situated in cellular membranes, regulating water homeostasis and cell volume. Moreover, they have been shown to play a pivotal role in cell migration and membrane protrusion formation providing a means for the plasma membrane to separate from the cytoskeleton allowing actin elongation into the water filled space to stabilize the protrusion. AQP9 is the main aquaporin in human immune cells. Using live cell microscopy we observed a cell-area change in 3O-C₁₂-HSL stimulated macrophages. Furthermore mRNA and protein analyses revealed a dose-dependent increase in the AQP9 expression. After infection of *P. aeruginosa* PAO1 both the expression and cellular distribution of AQP9 were affected. Thus, with high resolution imaging we found an evenly smooth distribution and high intensity of AQP9 in the wild type but not in the lasI⁻/rhlI⁻

infected cells. All together, studying the inter-kingdom interaction between *P. aeruginosa* and human immune cells can elicit an array of eukaryotic responses both at the transcriptional and cellular structure levels strengthening the importance of water homeostasis and thus a role of AQP_s in infection and inflammation.

P1567

Board Number: B1070

Mucosa-associated *E. coli* as an IBD exposome prolongs epithelial NF- κ B activation via macrophage inhibitory cytokine 1.

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Commensal bacterial community shifts in the pathogenic colonic environment and chronic colonization of mucosa-associated *Escherichia coli* (MAEC) has been linked to chronic colonic inflammatory diseases. Enteropathogenic *Escherichia coli* (EPEC) is one of commonly identified MAEC in inflammatory bowel disease patients. The aim of this study is to address the contribution of MAEC colonization to human inflammatory signals such as nuclear factor kappa B (NF- κ B). The present study was conducted to investigate the prolonged epithelial responses to persistent EPEC infection via NF- κ B activation. EPEC infection led to sustained activation of NF- κ B signal in mouse intestinal epithelial cells in vivo and in vitro, which was positively associated with a type III secretion system, whereas early NF- κ B is regulated. Moreover, prolonged NF- κ B activation was found to be a part of macrophage inhibitory cytokine 1 (MIC-1)-mediated signaling activation, a novel link between NF- κ B signaling and infection-associated epithelial stress. Functionally, both EPEC-induced MIC-1 and NF- κ B signaling mediated epithelial survival by enhancing the expression of cyclin D1, a target of NF- κ B. In summary, the results of the present study suggest that MIC-1 serves as a mediator of prolonged NF- κ B activation, which is critical in maintaining gut epithelial integrity in response to EPEC-induced injuries (This work was supported by the Basic Science Research Program through the National Research Foundation of Korea, funded by Ministry of Education, Science and Technology Grant 2012R1A1A2005837, and a grant from the Korean Health Technology Research and Development Project, Ministry of Health and Welfare, Republic of Korea (HI13C0259)).

P1568**Board Number: B1071****The E3 ubiquitin ligase MABP negatively regulates the MAVS-mediated innate immune response.**Y. Yoo¹, H. Cho², Y. Park²;¹Ajou University, SuWon, Korea, ²Ajou University, Suwon, Korea

Mitochondrial antiviral signaling (MAVS) is an essential adaptor protein in innate RIG-I-like receptor signaling pathways and tightly regulated to ensure a rapid response to pathogens and to limit host immunopathology. Mitochondria act as a crucial platform for MAVS activation and inhibition of MAVS is also essential to limit excessive harmful immune response; however, how these signaling pathways are integrated at the mitochondria remains unclear. Here, we report that MABP is a negative regulator of MAVS. MABP deficiency in bone marrow-derived macrophages, Raw 264.7 and THP-1 resulted in increased type I interferon production and a marked reduction in viral replication of influenza A and vesicular stomatitis virus (VSV). MABP-deficient mice exhibited reduced susceptibility and enhanced survival following VSV infection. MABP binds to MAVS upon viral infection, promoting proteasome-mediated degradation of MAVS via Lys48-linked polyubiquitination of Lys7 and Lys500 residues of MAVS. Together, these findings identify MABP as a novel regulator of host antiviral immunity that ensures timely degradation of activated MAVS.

P1569**Board Number: B1072****Anti-chaperone activity of human α -defensin HNP1 against bacterial toxins.**E. Kudryashova¹, R. Quintyn¹, S.M. Seveau², W. Lu³, V. Wysocki¹, D.S. Kudryashov^{1,4};¹The Department of Chemistry and Biochemistry, The Ohio State University, Columbus, OH,²Microbiology, The Ohio State University, Columbus, OH, ³The Department of Chemistry andBiochemistry, University of Maryland, Baltimore, MD, ⁴Molecular and Cellular Developmental Biology Program, The Ohio State University, Columbus, OH

Defensins are short cationic, amphiphilic, cysteine-rich peptides that constitute the front line immune defense against various pathogens. In addition to exerting direct antibacterial activities, defensins inactivate several classes of unrelated bacterial exotoxins. To date, no coherent mechanism has been proposed that would explain defensins' enigmatic efficiency towards various toxins. We show that binding of α -defensin HNP1 to affected bacterial toxins causes their local unfolding, potentiates their thermal melting and precipitation, exposes new regions for proteolysis, and increases susceptibility to collisional quenchers, without causing similar effects on tested mammalian structural and enzymatic proteins. We propose that protein susceptibility to inactivation by defensins is contingent to their thermolability and conformational plasticity and that the defensin-induced unfolding is a key element in the general mechanism of toxin inactivation by human α -defensins.

P1570**Board Number: B1073****Chemical Biology to probe the molecular mechanisms of innate immune activation.**C.L. Grimes¹, J. Melnyk¹;¹Chemistry and Biochemistry, University of Delaware, Newark, DE

The innate immune system is charged with a tremendous task: it must deal with bacterial threats and the trillion of commensal bacteria that occupy the human body. In order to deal with this bacterial load, the innate immune system uses over twenty innate immune receptors to work in concert with each other to generate a proper immune response. However, when one of these receptors is broken, tremendous consequences can ensue. This is the case in Crohn's Disease; the receptor, Nod2 is mutated and is not able to properly respond to bacteria. Recently our laboratory has developed in vitro assays to measure the binding between Nod2 and its ligand, fragments of bacterial cell wall. We have shown that in vitro Nod2 binds to muramyl dipeptide with nanomolar affinity. However, this interaction has not been shown to occur in vivo. In order to determine if Nod2 interacts with this bacterial cell wall ligand in a cellular context, we have used synthetic organic chemistry to build bacterial cell wall fragments that contain a photo-activatable cross linker. We will describe the synthesis and biological activity of these derivatives. In addition, we will describe the biological targets that these cross-linking derivatives identified in preliminary experiments. It is our hope that by the development of the proper probes, we can use our background in chemical biology to understand the molecular recognition motifs used by nature to sense bacterial cell wall fragments.

P1571**Board Number: B1074****Ribosomal Inactivation Disrupts Gut Innate Immunity and Barrier Integrity in *Caenorhabditis elegans* through p38/MAPK Signaling Pathway.**J. PARK¹, Y. Moon²;¹Department of Biomedical Sciences, BB21 project, Pusan National University School of Medicine, Yangsan, Korea, ²Department of Biomedical Sciences, Pusan National University School of Medicine, Yangsan, Korea

Ribosomal inactivation has been known as one of exogenous etiological factors of experimental or human inflammatory bowel disease (IBD). Mechanistically, ribosomal inactivation triggers pro-inflammatory responses which may mediate the disruption of epithelial barrier and subsequently allows translocation of luminal bacteria as the crucial inflammatory agent. To provide these mechanistic evidences of the gut barrier-based pathogenesis of environmental IBD, we used gut exposure model in *Caenorhabditis elegans* since the nematode living in soil has a simple innate defense system with gut epithelial barrier

against luminal antigens including commensal bacteria. Based on our observation that *C. elegans* under the mucosal stress by ribosomal inactivation showed shorter life span than the control group, the molecular mechanism was addressed in association with gut barrier integrity. In term of gut bacteria, mucosal ribosomal inactivation increased gut load of ingested bacteria and p38/MAPK deficiency allowed more microbial exposure in the gut, indicating the protective roles of p38-linked gut defense. Moreover, various lysozymes, the crucial innate immune mediators, were also interfered by ribosomal inactivation, which contributed to more bacterial exposure to the nematode gut lumen. The ribosomal inactivation-disrupted gut integrity was also confirmed by barrier staining and monitoring of microbial translocation into the sub-epithelial parts of gut in *C. elegans*. All of these events were highly dependent on the p38/MAPK signaling pathway. Taken together, mucosal ribosomal inactivation disturbed innate immunity in the intestinal barrier of *C. elegans*, which led to more microbial infection and subsequently shorter lifespan. The present model can be associated with the mucosal disorder under ribosome-inactivating stress, a potent environmental trigger of human IBD (This work was supported by the Basic Science Research Program through the National Research Foundation of Korea, funded by Ministry of Education, Science and Technology Grant 2012R1A1A2005837, and a grant from the Korean Health Technology Research and Development Project, Ministry of Health and Welfare, Republic of Korea (HI13C0259)).

P1572

Board Number: B1075

Dictyostelium discoideum TirA mediates signaling in response to bacterial prey.

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Cells of the innate immune system use pattern recognition receptors (PRRs) to detect conserved molecular motifs on a wide variety of microbial pathogens. The best characterized PRRs are the Toll-like receptors (TLRs), which utilize toll/interleukin-1 receptor (TIR) domains to initiate signaling responses. Among the signaling pathways activated downstream of mammalian TLRs are NF- κ B, caspase and MAPK-mediated pathways, as well as the generation of reactive oxygen species (ROS). The social amoeba *Dictyostelium discoideum* phagocytizes bacteria for nutritional purposes. While its genome does not encode for full-length TLRs, the model organism does express several TIR domain-containing proteins, including *D. discoideum* TirA, which previously has been shown to play a role in the growth of *D. discoideum* in the presence of Gram-negative bacteria. Furthermore addition of lipopolysaccharide (LPS), a component of the Gram-negative cell wall has been shown to enhance *D. discoideum*'s bactericidal activities in a TirA- and ERK-dependent manner. While *D. discoideum* does not encode for NF- κ B or caspases, exposure of *D. discoideum* to bacteria does result in the activation of MAPK-ERK signaling pathways as well as the generation of ROS. Here we find that both ERK phosphorylation, as well the generation of ROS, are reduced in TirA-deficient cells as compared with WT cells in response to Gram-negative, but not Gram-positive bacteria. In contrast, ERK phosphorylation in response to folic acid does not appear to be greatly affected by the absence of TirA. We are currently investigating the

role of additional signaling molecules in the response of *D. discoideum* to Gram-negative bacteria. In addition to these procedures we are also attempting to solve the structure of *D. discoideum* TIR domains through the use of X-ray crystallography. By obtaining this structural information new insights into the conserved mechanisms of TIR domain signaling in may be revealed.

P1573

Board Number: B1076

Altered N-Glycan profile play important roles on growth and morphogenesis of *Paracoccidioides brasiliensis* and on the biological activities of yeast proteins.

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The fungus *Paracoccidioides brasiliensis* is a human pathogen that causes paracoccidioidomycosis, the most prevalent systemic mycosis in Latin America. The cell wall of *P. brasiliensis* is a network of glycoproteins and polysaccharides, such as chitin and glucan, performing several functions. N-linked glycans are involved in glycoprotein folding, intracellular transport, secretion, and protection from proteolytic degradation. We investigated the influence of tunicamycin (TM)-mediated inhibition of N-linked glycosylation on N-acetyl- β -D-glucosaminidase (NAGase), α - and β -(1,3)-glucanases and on α -(1,4)-amylase in *P. brasiliensis* yeast and mycelium cells. The underglycosylated yeasts were smaller than their fully glycosylated counterparts and exhibited a drastic reduction of cell budding, reflecting impairment of growth and morphogenesis by TM treatment. The intracellular distribution in TM-treated yeasts of the *P. brasiliensis* glycoprotein paracoccin was investigated using highly specific antibodies. Paracoccin was observed to accumulate at intracellular locations, far from the yeast wall. Paracoccin demonstrated lower NAGase activity when underglycosylated, although no difference was detected between the pH and temperature optimums of the two forms. Murine macrophages stimulated with underglycosylated yeast proteins produced significantly lower levels of TNF- α and NO. Moreover, incubation with TM did not alter α - and β -(1,3)-glucanase activity in yeast and mycelium cell extracts. In contrast, NAGase and α -(1,4)-amylase activity was significantly reduced in underglycosylated yeast and mycelium extracts after exposure to TM. In spite of its importance for fungal growth and morphogenesis, N-glycosylation was not required for glucanase activities. This is surprising because these activities are directed to wall components that are crucial for fungal morphogenesis. On the other hand, N-glycans were essential for α -(1,4)-amylase activity involved in the production of malto-oligosaccharides that act as primer molecules for the biosynthesis of α -(1,3)-glucan. Our results suggest that reduced fungal NAGase and α -(1,4)-amylase activity affects cell wall composition and may account for the impaired growth of underglycosylated yeast and mycelium cells. Taken together, the impaired growth and morphogenesis of tunicamycin-treated yeasts and the decreased biological activities of underglycosylated fungal components suggest that N-glycans play important roles in *P. brasiliensis* yeast biology.

P1574**Board Number: B1077****Proteome analysis reveals differential expression of proteins in macrophages J774A.1 infected with *Leishmania amazonensis*.**J.C. Buitrago-Gutiérrez^{1,2}, S.S. Novoa-Herrán¹, R.A. Rodríguez-Castro¹, M.Y. Sánchez-Gómez¹, M. Camacho^{2,3};¹Química, Universidad Nacional de Colombia, Bogotá D.C., Colombia, ²Centro Internacional de Física, Bogotá D.C., Colombia, ³Biología, Universidad Nacional de Colombia, Bogotá D.C., Colombia

The phenomena described by our group that characterizes the impact of *Leishmania* infection on the electrical properties of infected macrophages, the decrease in the level of Kir2.1 expression in *Leishmania amazonensis* infected macrophages, revealed that the protein profile is different compared to non-infected macrophages and prompted us to study this in more detail. The macrophage-like cell line J774A.1, *L. amazonensis* promastigotes as well as infected macrophages were culture in vitro and harvested by centrifugation to obtain total protein extracts in RIPA buffer. The protein concentration was determined by densitometry and protein separated in 1D and 2D electrophoresis (2DE). Proteins were focused in equilibrated IPG-strips of 7cm pH linear (Bio-Rad) within a range between 3 to 10, and then separated in 10% SDS-PAGE gels which were stained with colloidal Coomassie Brilliant Blue G-250, before documentation in VersaDoc™ imaging system (Bio-Rad) for image analysis with Melanie 7.0 software (Swiss Institute of Bioinformatics). The initial gels show that most of the spots detected for non-infected and infected macrophage total extracts were in the pH range 5 to 8. Thus, this pH range was chosen for the 2DE reference maps. The analysis showed high reproducibility in terms of spot number, relative position and intensity between gel replicas of a particular treatment (e.i non-infected). Approximately 205 and 178 spots were detected for non-infected and infected macrophage total extracts, respectively. In order to find protein expression differences between samples, the statistical test ANOVA was used and spots with p-values below 0.5 were selected. Statistically significant differences in the expression level of the spots was found in 58% of the cases. These differences are related in a greater proportion to unique expression in any of the two classes: 34.2% to non-infected and 16.9% to infected macrophages. Quantitative changes in the expression level of shared spots was also found where in 2.6% of them the expression level was higher in non-infected, while 4% of the spots the expression level decreased in non-infected macrophages. This is the first study in which the differential expression of protein level from macrophages J774A.1 infected with *Leishmania amazonensis* is achieved using two-dimensional electrophoresis and revealing differential expression of proteins attributable to infection.

P1575**Board Number: B1078****Ralstonia solanacearum provokes gene expression changes in Arabidopsis roots and targets different regions of the plant cell.**R.M. Mitra¹, A. Duncan¹, W. Truman², J. Lu¹, R. Chen¹, D. Mishkind¹, E. Matsuda¹, J. Glazebrook², F. Katagiri²;¹Biology Department, Carleton College, Northfield, MN, ²Plant Biology Department, Carleton College, Northfield, MN

Bacterial wilt, caused by *Ralstonia solanacearum*, is one of the most devastating diseases of plants worldwide affecting hundreds of plant species including crops such as potato and tomato. *R. solanacearum* can persist in moist soils and invades roots via wounds or sites of lateral root emergence, which provide access to the xylem tissue. In the xylem, the pathogen multiplies to high densities, inhibiting water flow and causing the entire plant to wilt.

In order to evade detection and repel plant defenses, phytopathogenic bacteria often introduce a suite of effector proteins directly into the plant cell using a type III secretion system. Approximately 95 type III effectors have been identified across members of the *Ralstonia* species complex, 32 of which are found in nearly all of the sequenced strains. Approximately half of these conserved effectors are not present in other bacterial pathogens, suggesting that *R. solanacearum* targets novel pathways in the plant.

We are using two approaches for studying the *Ralstonia*-plant interaction. First, we are characterizing the localization of conserved *R. solanacearum* effector proteins in planta with the goal of identifying plant targets that may play important roles in plant defense. Second, we are examining the plant transcriptional response to bacterial inoculation, as plants have evolved molecular mechanisms to recognize the presence or activity of specific bacterial effector proteins, and activate plant defense pathways. We have identified a group of genes that are differentially expressed in roots in response to *R. solanacearum* but not in leaves in response to root inoculation. Several of these genes are also responsive to the plant hormones auxin and cytokinin, methyl jasmonate and abscisic acid suggesting a role for these hormones in bacterial wilt disease. Together, these studies should provide information about the interface between the bacterial and plant cell during bacterial wilt disease.

Digestive and Excretory Organs

P1576

Board Number: B1080

Epithelial IKK β signaling activates esophageal inflammation and angiogenesis via GM-CSF.

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Background: Deregulation of IKK β and/or NF κ B activities is associated with different inflammatory diseases and with various forms of cancer, including reflux esophagitis. **Objective:** To delineate the role of epithelial IKK β signaling in the modulation of the esophageal microenvironment. **Methods:** The *ED-L2* promoter of Epstein-Barr virus was used to drive *Cre* expression, producing overexpression of constitutively active IKK β (IKK β ca) in mouse esophageal epithelial cells but not inflammatory cells or surrounding stroma. Human esophageal keratinocytes with overexpression of constitutively active IKK β and their conditioned media were used for *in vitro* studies of gene regulation, tube formation, and endothelial cell migration. **Results:** *ED-L2/Cre;Rosa26-SFL-IKK β ca (L2/IKK β ca)* mice had alterations not only in esophageal epithelia but also in the surrounding microenvironment. At 1 month of age, *L2/IKK β ca* mice had mild hyperplasia. By 1.5 month of age, esophageal epithelia of *L2/IKK β ca* mice became irregular with the presence of a CD45 positive inflammatory infiltrate. Furthermore, we observed increased microvessel density in *L2/IKK β ca* mice at 1.5 and 3 months of age. To identify the pro-angiogenic factors expressed by esophageal epithelial cells in response to IKK β activation, we performed mouse PCR arrays on epithelial scrapings from 1 month-old *L2/IKK β ca* mice. We identified Granulocyte-macrophage colony-stimulating factor (GM-CSF) as a critical inducer of angiogenesis. *L2/IKK β ca* mice had a 7-fold increase in *GM-CSF* expression. Human esophageal keratinocytes with overexpression of constitutively active IKK β had increased GM-CSF mRNA expression and protein secretion. The expression and secretion of GM-CSF was critical for angiogenesis as neutralization of GM-CSF impaired endothelial functions *in vitro*, including HUVEC cell migration and tube formation. We are currently examining the effects of a GM-CSF blocking antibody *in vivo*. **Conclusions:** Constitutive activation of IKK β in esophageal epithelial cells results in early changes in inflammation and the esophageal microvasculature via GM-CSF.

P1577**Board Number: B1081****Establishment of two novel portal myofibroblast RGF and RGF-N2 cell lines from rat liver and functional characterization.**M. Fausther^{1,2}, J.R. Goree^{1,2}, E.G. Lavoie^{1,2}, J.A. Dranoff^{1,2};¹Internal Medicine, University of Arkansas Medical Sciences, Little Rock, AR, ²Research Service, Central Arkansas Veterans Administration Health Center, Little Rock, AR

Background and Rationale: Activated portal fibroblasts, along activated hepatic stellate cells, are increasingly recognized as major sources of scar-forming myofibroblasts during hepatic fibrosis, particularly in cholestatic liver injury settings. However, in contrast to well-characterized hepatic stellate cells, portal fibroblasts are understudied and remain poorly defined. Additionally, isolation of rodent portal fibroblasts for extensive functional studies remains technically challenging due to low cell numbers, reduced viability, variable purity, and limited growth capacity. To remediate this issue, two polyclonal cell lines, RGF and RGF-N2, were established from primary portal fibroblasts isolated from adult rat livers, and subjected to culture activation and subsequent SV40-mediated immortalization. Particularly, Ntpdase2/Cd39L1-sorted primary rat portal fibroblasts were used to generate the RGF-N2 cell line. **Main results:** First, gene screening indicates that primary (non-immortalized) culture-activated portal fibroblasts and both immortalized RGF and RGF-N2 cells are positive for myofibroblast markers such as, alpha smooth-muscle actin, type I collagen, tissue inhibitor of metalloproteinases 1, Ntpdase2, Cd73, and “portal fibroblast”-specific elastin, while negative for “hepatic stellate cell”-specific desmin and Irat, as determined by RT-PCR. Expression of alpha smooth-muscle actin, elastin, SV40 large antigen, Ntpdase2 and Cd73 proteins was confirmed in both RGF and RGF-N2 cell lines by immunofluorescence. Moreover, RGF and RGF-N2 cells are negative for “hepatocyte”-specific albumin and “macrophage”-specific F4/80 markers, although positive for “cholangiocyte/bile duct cell”-specific cytokeratin marker, as determined by RT-PCR. Absence of cytokeratin expression was established by immunoblot. Second, preliminary transfection assays using GFP expression vector and Fugene reagent show that DNA transfer is achievable in RGF and RGF-N2 cells. Lastly, the proliferation rate of immortalized Mz-Cha-1 cholangiocytes is decreased during co-culture with RGF and RGF-N2 cells in a bromodeoxyuridine uptake assay, a feature that has been also described for primary culture-activated portal fibroblasts.

Conclusion: Immortalized rat portal myofibroblast RGF and RGF-N2 cell lines express markers typical of myofibroblasts deriving from activated portal fibroblasts, are suitable for DNA transfection, and can inhibit bile ductular proliferation. Both RGF and RGF-N2 cell lines represent novel and useful in vitro cellular models for the functional studies of portal (myo)fibroblasts and their contribution to the progression of liver fibrosis.

P1578**Board Number: B1082****Determination of signature microRNAs in experimental liver fibrosis by miRnome profiling of circulating exosomes.**L. Chen¹, R. Chen¹, D.R. Brigstock^{1,2};¹Center for Clinical and Translational Research, Nationwide Children's Hospital, Columbus, OH,²Department of Surgery, The Ohio State University, Columbus, OH

Background: Hepatic fibrosis is a serious pathology that compromises normal hepatic structure and function and is a harbinger of cirrhosis, hepatocarcinoma, and end-stage liver disease. Current methods of fibrosis assessment are limiting and do not allow progression or resolution to be accurately determined. Since a major goal it to establish non-invasive protocols that are amenable to longitudinal analysis in the dsame individuals, we have analyzed the miR content of circulating exosomes to identify potential fibrosis markers. Exosomes are membranous nanovesicles that arise by inward budding of multivesicular bodies and are released extracellularly when these multivesicular bodies fuse internally with the plasma membrane; thereafter exosomes traverse the intercellular spaces and may be taken up by neighboring cells. Exosomes contain a complex mixture of miRs, mRNAs and proteins that is a fingerprint of their producer cells, offering the opportunity to establish signature molecular profiles in health and disease.

Methods: Serum exosomes were isolated (PureExo Exosome Isolation Kits) from the circulation of mice after 1 or 5 weeks of treatment with CCl₄, or from oil-treated controls, with liver injury/fibrosis confirmed histologically. Exosomal RNA from 1ml of pooled serum (5 mice; 200µl/mouse) was profiled using a mouse miRnome miR PCR Array, allowing analysis of the 940 best characterized miRs in the mouse miRnome. Differentially expressed miRs were confirmed and/or further evaluated by qRT-PCR of exosomal RNA independently obtained at 1-, 4- or 5-weeks of CCl₄ administration (n=5).

Results: Microarray analysis revealed that, as compared to their respective oil controls, there were significant alterations in the expression of numerous miRs after either 1- or 5-wks of CCl₄ treatment. After 5 weeks of CCl₄ treatment, miR-7a, -21, -22, -24, -34a, -155, or -195 were all upregulated and miR-27a, -192, -214, or -377 were all downregulated and mirrored the respective changes in hepatic expression that has been previously reported for each of these miRs. Additionally, upregulation of exosomal miR-26b or -122 and down-regulation of miR-9 or -196b were noted as consistent changes that highlighted these additionalmiRs as potentially novel fibrosis markers that have not previously been identified in earlier studies. Many of these miRs exhibited individually distinct patterns of expression during fibrosis progression highlighting their potential utility for assessment of different fibrotic stages.

Conclusions: Dynamic changes occur in the miR content of circulating exosomes during experimental hepatic fibrosis showing that fibrosis progression and severity is amenable to minimally-invasive assessment through determination of signature exosomal miRs.

P1579**Board Number: B1083****Role of p217 and p290 in activation of INS-R on salivary glands of diabetic mice.**R.E. da Silva¹, R.O. Netto¹, M.J. de Barros¹, T.M. Augusto^{2,3}, L.A. Peroni¹, E.J. Caldeira¹;¹Morphology and Basic Pathology, Faculty of Medicine of Jundiaí, Jundiaí, Brazil, ²Clinical Department, Faculty of Medicine of Jundiaí, Jundiaí SP, Brazil, ³Structural and Functional Biology Department, State University of Campinas, Campinas SP, Brazil

Various studies have suggested that the glutamic acid decarboxylase (GAD) and its proteins play a critical role in type 1 diabetes. However, questions remain about the total efficacy of this therapy. The objective of this study was analyzed the activity of peptides p217 and p290 of GAD, in the possible re-establishment of salivary organs of diabetic mice. A total of 21 mice were divided in 3 series: I: formed by 7 Balb/C (control mice); II: formed by 7 diabetic NOD mice; and III: with 7 diabetic NOD mice. The group III received the peptide p290 associated to p217 (200µg diluted in 100µl of Incomplete Freund's Adjuvant). The animals (I and II) were subjected to intraperitoneal injections of saline solution and in all groups, the glucose levels were observed during the twenty-one days and salivary gland specimens were obtained for the fluorescence microscopy analysis of INS-R (Ethical Process Number: 304/11). Peptide therapy promoted reduction in glucose levels and the re-establishment of insulin receptors (INS-R) in both salivary organs. The peptide vaccine, especially when combined the p217 and p290, brought a major interaction between insulin and receptors. Thereby, this therapy contributed to the glucose balance and activated the α -subunits of insulin receptors, showing the regression of damage in the salivary organs. Support: FAPESP #2012/18012-2, CAPES, CNPq, and NAPED/FMJ.

P1580**Board Number: B1084****Human placenta-derived mesenchymal stem cell promote hepatic regeneration on BDL-injured rat hepatic failure model via dual function of phosphatase of regenerating liver-1.**J. Choi¹, S. Lim^{1,2}, J. Park³, I. Oh⁴, G. Kim¹;¹Dept. of Biomedical Science, CHA University, Seoul, Korea, ²Dept. of Biomedical Science, Cha University, Seoul, Korea, ³Dept. of Biomedical Sciences and Pharmacology, Seoul National University College of Medicine, Seoul, Korea, ⁴Catholic High Performance Cell Therapy Center, The Catholic University of Korea, Seoul, Korea

Hepatic failure is the inability of the liver to perform its normal function as well as irreversible severe disease. Placenta-derived mesenchymal stem cells (PD-MSCs) have been introduced as an attractive cells comparing to other MSCs. We previously reported that therapeutic effect of PD-MSCs on CCl₄-injured early cirrhosis rat model. However, it is still controversy the effect of PD-MSCs depend on animal models. Therefore, the objectives of this study were to analyze the effect of PD-MSCs on hepatic failure

by bile duct ligation (BDL), which is representative late stage of hepatic diseases. The survival ratio in a group with PD-MSCs transplantation and proliferation activities of hepatocyte isolated from rat models were significantly enhanced comparing to sham group. Otherwise, rat model engrafted PD-MSCs reduced the hepatic fibrosis compared with BDL until 2 weeks. PD-MSCs transplantation show anti-apoptotic effect via increasing Bcl2 and decreasing Bax and cleaved PARP expressions. Taken together, PD-MSCs transplantation could be enhanced liver regeneration in severe hepatic failure model through anti-fibrotic and anti-apoptotic effects. Therefore, the findings provide fundamental background for understanding of PD-MSCs effect in hepatic diseases as well as support to application of cell therapy using PD-MSCs.

Blood Cells and Vessels

P1581

Board Number: B1085

HIV-1 Antiretroviral, Efavirenz Stimulates Cathepsin Protease Activity in Macrophages: Implications for HIV-associated Atherosclerosis.

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Approximately 1.2 million individuals in the United States are living with HIV. Antiretrovirals (ARV) effectively manage viral loads, both preventing progression to AIDS and enhancing patient survival. However, reports indicate that people with HIV exhibit an increased risk for developing atherosclerosis, a form a cardiovascular disease characterized by lipid and macrophage-filled plaque formation and arterial remodeling. Recent research implicates ARVs in the development of atherosclerosis, as efavirenz correlates with endothelial dysfunction and elevations in low-density lipoprotein. Yet, the mechanisms underlying this association are not completely understood. In preliminary studies, we found that AZT increased cysteine cathepsin activity in arteries in a mouse model. These cathepsins are potent proteases, known to promote vessel remodeling via collagen and elastin degradation. Therefore, we hypothesize that efavirenz contributes to atherosclerotic vascular remodeling by increasing cathepsin protease activity. To test this, we exposed Thp-1 monocytes or monocyte-derived macrophages to efavirenz (25 μ M) for 24 hours. Cells were then collected and analyzed for cathepsin protein and active enzyme by immunoblot and multiplex cathepsin zymography, respectively. Our results demonstrate that efavirenz exposure causes a 50% decrease in monocyte levels of active cathepsins (n=6; p<0.05), which was reflected by significant reductions in their ability to degrade elastin (n=4, p<0.05). Efavirenz also reduces cathepsin mRNA and protein expression, but increases the levels of cystatin C, the extracellular protein cathepsin inhibitor. Conversely, in monocyte-derived macrophages, efavirenz exposure induced 2-fold increases in cathepsins V, S, and L expression and activity (n=6; p<0.05). Moreover, western blot analyses indicate that efavirenz-induced alterations in monocyte and macrophage cathepsin activity are mediated by JNK signaling. Overall, our findings demonstrate that efavirenz increases macrophage-

derived cathepsin activity. These efavirenz-induced elevations in cathepsin degradation may promote vessel remodeling and accelerate atherosclerosis development and progression.

P1582

Board Number: B1086

Stress reticulocytes, CD 36 and CD 49d in SSFA2 homozygous sickle cell black patients followed in Abidjan, Côte d'Ivoire.

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The complex physiopathology of sickle cell disease involves many factors, among them adhesion molecules such as the $\alpha 4\beta 1$ integrin or CD 49d and CD36. These antigens are found on immature reticulocytes or stress reticulocytes. We investigated whether there were links between the clinical manifestations and the presence of stress reticulocytes and the expression of CD 36 and CD 49d. We evaluated 60 SSFA2 homozygous sickle cell in patients (30 in painful crisis and 30 in steady state) with complete blood count, the presence of total reticulocytes, total stress reticulocytes, and CD 36 and CD49d antigens with immunophenotyping by flow cytometry. Out of the 308, 845/uL reticulocytes, 48.2% and 42.5% expressed respectively CD 36 and CD 49d. In over 90% of patients, stress reticulocytes were found. The presence or absence of complications, or of moderate or severe clinical manifestations, was not correlated with the expression of CD36 and CD 49d. From a clinical point of view, there was no difference between subjects expressing or not the CD 36. Even if the level of stress reticulocytes, CD 49d + and CD 36 + reticulocytes were high, the clinical status was not correlated with the expression of adhesion molecules.

P1583

Board Number: B1087

Investigation of the Interactions between Antimalarial and Antiretroviral drugs.

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Malaria and AIDS are two infectious diseases that are prevalent in sub-Saharan Africa due to geographic overlap. Malaria, caused by the protozoan parasite *Plasmodium*, is transmitted to humans by the female *Anopheles* mosquito. The Human Immunodeficiency Virus (HIV), which can lead to AIDS, is a retrovirus that damages the immune system by destroying CD4 T-cells. It is transmitted both parentally and sexually. Previous research in our laboratory has shown that co-infected patients in Benin City, Nigeria taking both antiretroviral and antimalarial drugs, still had *Plasmodium* present in their bloodstream. We

have demonstrated that *P. falciparum* was the only species present in these patients at a 28.7% prevalence. We have also shown a lack of correlation between the levels of CD4 T-cells and *Plasmodium* in these patients suggesting no interaction between HIV and *Plasmodium*. Therefore, we hypothesized that antiretroviral drugs are inhibiting the antimalarial drugs. This was tested by performing a 3H-hypoxanthine incorporation assay in the presence of antimalarial and antiretroviral drugs, used individually and in combination. We found that the antimalarial drugs, sulfadoxine and lumefantrine, were inhibited by the antiretroviral drugs, zidovudine, lamivudine, and stavudine, respectively. We also found that stavudine had the most inhibitory effects on all of the antimalarial drugs, except for artemisinin.

P1584

Board Number: B1088

Functional analysis of the transcription factors specifically expressed in hematopoietic stem cells.

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Hematopoietic stem cells (HSCs) can generate blood cells throughout the lifespan of an organism owing to their self-renewal and multi-differentiation properties. However, the underlying mechanism for the maintenance of stemness of HSCs remains unclear. Hence, to clarify this mechanism, we focus on the transcription factors (TFs) that are specifically expressed in HSCs, and examine whether these TFs are involved with the maintenance of the undifferentiated state of HSCs. First, we performed RNA-seq of mouse HSCs (CD150+, CD34⁻, c-Kit+, Sca-1+, and Lineage marker⁻ cells), hematopoietic progenitor cells (HPCs; CD34+ c-Kit+, Sca-1+, and Lineage marker⁻ cells), and cultured HSCs. Thereby, we extracted 11 HSC-specific transcription factors (11 HSTFs) that seem to have the potential for the involvement with maintaining stemness of HSCs. Therefore, to determine whether these factors actually affect the undifferentiated state of HSCs/ HPCs, we prepared 11 of retroviral vectors for the transfection of each HSTF, and analyzed HPCs transduced with the cocktail of these retroviral vectors. These HPCs retained their proliferative activity for 32 days in vitro, and cells with c-Kit+, Sca-1+, lineage marker⁻ (KSL) phenotype, which is a cell surface lineage marker pattern specific to HSCs/HPCs, were constantly observed during long-term culture. In contrast, HPCs transduced with the control retroviral vector exhibited significantly decreased proliferation at approximately 14 days after the culture, resulting in the impossible passage at approximately 20 days. In addition, the transduction of the 11 HSTFs not only contributed to the maintenance of KSL phenotype in HPCs, but also allowed some c-Kit+, Sca-1⁻, lineage marker⁻ cells, which are regarded to be more differentiated than KSL cells, to gain the KSL phenotype again. In contrast, this phenomenon was never observed in c-Kit+, Sca-1⁻, lineage marker⁻ cells transduced with the control retroviral vector. Furthermore, KSL cells induced from c-Kit+,

Sca-1-, lineage marker— cells by 11 HSTFs-transduction also retained not only proliferation activity but also KSL phenotype during long-term culture. These results indicate that these 11 transcription factors, individually or in combination, contribute to the maintenance of the undifferentiated state of HPCs, thus suggesting that these factors are involved in the maintenance of stemness of HSCs. Therefore, we have now filtered the transcription factors that actually induced these observed phenomena, and have simultaneously examined whether the factors affect stemness of HSCs.

P1585

Board Number: B1089

Pseudomonas aeruginosa induces the production and release of a cytotoxic factor from pulmonary endothelial cells.

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Pseudomonas aeruginosa infection causes pneumonia that can progress to sepsis and acute respiratory distress syndrome. Patients who survive the initial infection oftentimes succumb to *Pseudomonas* months later, secondary to progressive end organ dysfunction that occurs after the bacteria have been killed. These data suggest that a toxic substance is produced during the infection process that contributes to progressive demise. We have tested this possibility in vitro using cultured rat primary microvascular endothelial cells (PMVECs) and pulmonary artery endothelial cells (PAECs). *P. aeruginosa* uses a type III secretion system to inject various exoenzymes into cells during infection processes. PMVECs were infected with *P. aeruginosa* and media were collected, filter sterilized, and then added to naïve PAECs and PMVECs. The inoculated cells died within 12-24 hrs following addition of the media. The importance of infection of the endothelial cells was demonstrated when the PcrV strain, a type of *Pseudomonas* that lacks a functional type III secretion system, was used. PAECs treated with media collected from PMVECs that had been inoculated with PcrV bacteria survived. Similar results were obtained in a Matrigel vessel formation assay. Cells treated with medium collected from *P. aeruginosa* infected cells failed to form vessels while control cultures formed numerous vessels. The substance released from infected PMVECs resists DNase and RNase digestion, and is resistant to boiling. The substance can be precipitated from culture supernatants using 50% ammonium sulfate. RNA analyses determined that PMVECs express various known and suspected pathologic proteins including prion protein, tau, alpha synuclein, and amyloid precursor protein, and immunoblot analyses have demonstrated that several of these proteins are released from PMVECs during *Pseudomonas* infection. Studies are in progress to identify the cytotoxic substance released from PMVECs following infection with *P. aeruginosa* and to establish possible roles for this substance in clinical outcomes.

P1586**Board Number: B1090****Endothelial to Mesenchymal Transition (EndoMT) leads to a loss of normal endothelial cell function and may contribute to the development of pulmonary arterial hypertension.**R.B. Good¹, A. Gilbane¹, S. Trinder¹, C. Denton¹, G. Coghlan², D. Abraham¹, A.M. Holmes¹;¹Rheumatology and Connective Tissue Diseases, UCL- Royal Free hospital, UCL - Royal Free Campus, Rowland Hill St, United Kingdom, ²Royal Free Hospital NHS Foundation Trust, National Pulmonary Hypertension Service, London, United States**Background**

Endothelial cells form a biological exclusion barrier actively regulating the exchange of macromolecules, solutes and cells, between the blood and the surrounding tissues. Expansion of vascular mesenchymal cells and endothelial dysfunction are cardinal features that contribute to vascular remodelling in pulmonary arterial hypertension PAH. Recent studies suggest that as well as resident mesenchymal cells, endothelial cells can undergo endothelial-mesenchymal transition (EndoMT) and acquire a mesenchymal phenotype, which can contribute to the expansion of mesenchymal cell populations. Here we sought to assess the capacity of pulmonary artery endothelial cells to undergo EndoMT and assess the cellular effects and determine the prevalence of EndoMT in the pulmonary vasculature of PAH patients and pre-clinical models of PAH.

Methods

EndoMT was induced in human PAECs *in vitro* by exposure to TNF α , IL-1 β and TGF β in combination. Western blotting and immunofluorescence was used to quantify EndoMT: CD31, vWF, occludin, VE-cadherin, α SMA, calponin and collagen type 1 expression. Levels of pro-inflammatory secretion was quantified by MSD arrays on conditioned PAEC, EndoMT and HC and SSc-PAH fibroblast media. The capacity of EndoMT monolayers and mixed cultures comprising 1:10 EndoMT:PAECs cells to form exclusion barriers was assessed using trans-well permeability FITC-albumin assays. Using lung tissue from PAH and healthy control (HC) donors, and from the hypoxia/SU5416 pre-clinical murine model of PAH, the presence of EndoMT was assessed by immunofluorescence based on co-expression of vWF and α SMA.

Results

PAECs treated with TNF α , IL-1 β and TGF β exhibited significant morphological changes, loss of endothelial markers and gained expression of mesenchymal markers by day 6. There was a significant (P<0.01) 5-fold increase in permeability compared to PAECs alone. Consistent with this, EndoMT cells co-cultured with PAECs in a ratio of 1:10 led to 2.5-fold significant (P>0.05) increase in permeability. Assessment of pre-clinical models and clinical material from PAH patients for EndoMT demonstrated co-localisation of vWF and α SMA in \leq 5% of pulmonary arteries.

Conclusion

We demonstrate that EndoMT is present in patients and pre-clinical models of PAH. Induction of EndoMT *in vitro* leads to a loss of normal PAEC morphology and an enhanced secretion of pro-inflammatory chemokines. Furthermore EndoMT cells failed to form integral biological barriers and contributed to enhanced permeability of PAEC barriers. Collectively our data suggests that EndoMT may contribute to the loss of normal endothelium function and the development of PAH.

P1587

Board Number: B1091

Angiotensin II AT1 receptors blockers reduce cholesterol deposition in AGE-LDL induced foam cells via reverse cholesterol transport pathway.

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[Objective] AGE(Advanced Glycation Endproduct)-LDL, may be formed in DM (Diabetes mellitus) patients, reduces cholesterol export from the foam cell and enhances foam cell apoptosis. To elucidate the intracellular cholesterol traffic of foam cells, the relationship among AGE-LDL, FC-related foam cell apoptosis were investigated. HDL, potent molecule cholesterol uptake from atherosclerotic lesions, strongly associated with ABC transporter function of foam cells. To confirm disfunction of reverse cholesterol transport in AGE-LDL induced foam cells can be improved by **vascular statins** and **ARB** (telmisartan, losartan, irbesartan) treatment. **[Method]** Foam cells were prepared by J774 cells incubating with various human modified LDL for 24-72hr, then cultured up to 3-6 days in the presence of LPDS +/- HDL and ARB or statin. PC-FC complex and Apoptotic cells were detected by immunofluorescent microscopy using specific monoclonal antibodies or FITC-AnnexinV. FC rich lipid droplet can be detected by Filipin staining. **[Results & Discussion]** PC-FC Complex specifically recognized by atheroma specific monoclonal antibody (ASH1a/256C), induced in foam cells by Acetylated LDL(AcLDL), is corresponding to foam cell apoptosis via FC(free cholesterol)-rich lipid droplet formation. AGE-LDL is also inducing foam cells expressing FC-rich lipid droplets and PC-FC Complex, however, accumulation rate is very slow. HDL enhances FC reverse transport result in foam cell survive in AcLDL induced foam cells, whereas, HDL defects the FC-removing activity in AGE-LDL induced one. Vascular statin molecules (Simvastatin, Atrovastatin) had a potent activity of inducing ABC transporter and its translocation, avoid foam cell death and reduce FC-rich lipid droplet formation in AGE-LDL induced foam cells. HDL treatment might be affected cellular nCEase activity leading to cellular FC increase, in resulting foam cells expressed a little amount of FC-rich lipid droplets, PC-FC Complex. AGE-LDL and its metabolates may reflect on cellular FC transport including ABC transporters translocation. ARB was well known as PPAR ligand and its activity can be associated fatty acid metabolism. In ARB treated AGE-LDL induced foam cells, it was observed reducement of FC-rich lipid droplet formation, amount of lipid droplets and foam cell apoptosis. Telmisartan and irbesartan strongly reduce FC-rich lipid droplets in both AcLDL and AGE-LDL induced foam cell, however, losartan act only AGE-LDL induced foam cells, but no significant difference in AcLDL induced cells. These significantly relationship in AGE-LDL induced foam

cells, among the cellular FC increase, PC-FC complex formation, and foam cell apoptosis may reflect to development of atherosclerosis in DM patients. Contact me by; mmori@cis.ac.jp

P1588

Board Number: B1092

Hydroxychloroquine Binding Sites of Human Erythrocytes.

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Although hydroxychloroquine (HCQ) is used to use in the treatment of malaria, arthritis, and lupus for many years, the precise mechanism of their action remains unclear. In the present study, we investigated HCQ binding proteins in human erythrocyte membrane by using HCQ coupling Sepharose column chromatography. We prepared erythrocyte ghosts from healthy human blood removed white blood cells by cellulose column chromatography. The ghosts were dissolved in 50 mM Tris-HCl buffer, pH7.4 containing 0.15 M NaCl, 1 mM 2ME, 1 mM EDTA and 0.1% NP-40 (Buffer A). HCQ was coupled with epoxy activated Sepharose (HCQ-Sepharose). The erythrocyte membrane skeletal proteins, Band 3 (anion exchanger 1, AE1), spectrin, ankyrin, 80kDa protein 4.1R and protein 4.2 were eluted by 20 mM HCQ in Buffer A from HCQ-Sepharose. These proteins did not bind to plain Sepharose. NHE (Na⁺/H⁺ exchanger) 1 and CD47 did not bind to HCQ-Sepharose. Inside-out-vesicles (IOV) depleted of all peripheral proteins (pH11-IOV) and trypsin-digested pH11-IOV (T-pH11-IOV) were prepared according to methods previously described (Biochem. J. (2010) **432**:407-416). Band 3 in the IOVs and pH11-IOV bound to HCQ-Sepharose but not in T-pH11-IOV. Recombinant cytoplasmic N-terminal 43kDa domain of Band 3 bound to HCQ-Sepharose was eluted with 40 mM HCQ. These results indicate that the HCQ binding site of erythrocyte membrane is N-terminal cytoplasmic domain of Band 3 in human erythrocytes. Activity of lactate dehydrogenase (LD) is inhibited by chloroquine (JA Read et al. J. Biol. Chem. (1999) **274**:10213-10218). Activity of LD in the erythrocytes incubated with HCQ was decreased ~60% compared with non-treatment. That of cells treated with DIDS (4,4'-diisothiocyano-2,2'-stilbenedisulfonic acid) being Band 3 inhibitor following HCQ incubated was not changed. These results indicate that although major HCQ binding protein is Band 3 in human erythrocyte membrane, HCQ may not permeate thorough the Band 3. Finally, we investigated the effect of HCQ on the Band 3 activity. The activity was measured by the methods using dipoclinic acid and TbCl₂ described by Tu and Xu (Bioscience Reports (1994) **14**:159-169). The Band 3 activity of the HCQ treated ghost was ~20% reduced compared with none treatment. Taken together, HCQ binds to cytoplasmic N-terminal domain of Band 3 and reduces its activity in human erythrocytes.

POSTER PRESENTATIONS-Tuesday, December 09

Imaging Technologies, Single Molecule Imaging, and Super-Resolution 3

P1589

Board Number: B100

Hepatic DNA deposition and sensing: a new feature of drug-induced liver injury.

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Drug-induced liver injury (DILI) is an important cause of acute liver failure with limited therapeutic options. During DILI, necrosis and release of intracellular contents such as self-DNA may amplify liver inflammation and injury. Therefore, we aimed to determine whether DNA released from damaged hepatocytes accumulates within the necrotic liver and the impact of its recognition by the immune system. Treatment with different hepatotoxic drugs (paracetamol and thioacetamide, 20 and 70mM, respectively) caused DNA release to hepatocyte cytoplasm, which occurred in parallel with cell death *in vitro* (50% reduction in MTT metabolism). Administration of these drugs *in vivo* (600mg/kg) caused severe liver injury, shown by histology and increased serum transaminase levels (control = 43.5±7.6; paracetamol-treated = 3736±378.2). Surprisingly, using confocal intravital microscopy, we observed massive DNA deposition in liver necrotic areas (19% of total area), together with an intravascular DNA coating (25% of total area). In addition, liver injury led to a directional migration of neutrophils to DNA-rich areas, where they exhibited an active patrolling behavior. Analysis by flow cytometry showed that emigrated neutrophils expressed the DNA receptor TLR9 in their surface during injury, and these cells sensed and reacted to extracellular DNA by activating the transcription factor NF-κB (15-fold increase) and up-regulating the chemokine receptor CXCR2 (120-fold increase), measured by real-time PCR. DNA removal by intravenous injection of DNASE1 (1000 units; 6/6hours) or blockage of TLR9 receptor-mediated sensing (E6446; 100mg/kg) significantly reduced hepatic cytokine production (TNFα and CXCL1 levels; 80% and 50% respectively), liver neutrophil recruitment (50% inhibition) and hepatotoxicity (transaminase levels: DNASE-treated = 1489±288.3; TLR9 blocker-treated = 637±139) even if given 6 hours after the toxic challenge. Likewise, adoptive transfer of wild-type neutrophils to TLR9^{-/-} mice reversed the hepatoprotective phenotype otherwise observed in TLR9 absence. Conclusion: Hepatic DNA accumulation is a novel feature of DILI pathogenesis and blockage of DNA accumulation or recognition by the immune system may consist in a promising therapeutic option. Financial Support: CAPES, CNPq, FAPEMIG

P1590**Board Number: B101****Visual evidence for the direct interaction between MDA5 and IPS-1 proteins.**K. Deguchi¹, A. Yoshida¹, J.L. Gilmore¹, R. Yamaue^{1,2}, H. Kato^{1,2}, T. Fujita^{1,2}, K. Takeyasu¹;¹Graduate School of Biostudies, Kyoto University, Kyoto, Japan, ²Institute for Virus Research, Kyoto University, Kyoto, Japan

The front line of defense of a host cell against pathogenic invasion is the innate immune system. In the cytoplasm, several sensor proteins, including RIG-I-like receptors (RLRs), function against viral invasion. They recognize invading viral RNAs and, via the following several steps, produce type I interferons (IFNs) and IFN-inducible antiviral proteins. MDA5, one of the RLRs, is believed to be activated by binding specifically to long (>2000 base pairs) double-stranded RNAs (dsRNAs). The activated MDA5 is then assumed to interact with IPS-1 (also referred as MAVS/VISA/Cardif), a downstream factor localized on the outer mitochondrial membrane. At present, the molecular mechanism of the RNA—MDA5—IPS-1 signaling cascade has not been clarified. Here we established a method of investigating the protein-dsRNA/protein-protein interactions with a combination of biochemistry, molecular biology, and atomic force microscopy (AFM). The results obtained are:

Multiple MDA5 molecules bind to dsRNA, forming fiber-like structures on it.

MDA5 consists of three domains (C-terminal domain, helicase domain, and two CARDs), which can be recognized individually by AFM, and exists in an equilibrium between open and closed conformations.

In a mutant form of MDA5 (which constitutively activates the IFN pathway), the equilibrium is shifted to the closed form, suggesting that the closed form is active.

After ATP-hydrolysis of the fiber-like structures, the closed form of wild-type MDA5 is released, consistent with the idea that the closed form is active.

The constitutively active MDA5 mutant, many of whose molecules are closed, induces significantly more *in vitro* aggregation of IPS-1 than the wild type MDA5. The volume of such aggregate is 100~ times as large as that of a single molecule of IPS-1. This provides direct evidence that the closed MDA5 is active and that the activated MDA5 interacts directly with IPS-1.

From these results, we propose a new model of the RNA—MDA5—IPS-1 signaling cascade. The open MDA5 recognizes viral dsRNA by binding to it and forming fibrous structures. This process is accelerated by binding to ATP. The MDA5 molecules on dsRNA release themselves from it after ATPase-hydrolysis, and then interact with IPS-1 on the outer membrane of mitochondria.

P1591**Board Number: B102****Efficient neuron reconstruction with NeuTu.**R. Pearcy¹, D. Hunt², D. Jin³, N. Spruston²;¹University of Richmond, Richmond, VA, ²Janelia Research Campus, Ashburn, VA, ³Pennsylvania State University, State College, PA

Neurons have a broad range of morphologies that directly impact cellular function. Analyzing morphology can help discern the possible functions of specific cell-types and the mechanisms behind how cells process synaptic inputs, as well as helping to create accurate computational models of neurons. To study morphology, it is crucial to reconstruct neurons in an accurate and efficient manner. NeuTu is a novel software tool that has been recently developed with the goal of reducing the time and effort required for neuron tracing and reconstruction compared to the previously used manual methods. NeuTu processes tiles of bright-field image z-stacks of neurons and uses a semi-automatic tracing method to reconstruct neurites with accurate shapes and diameters. The neurites are represented in SWC format in a 3D viewer and are also displayed in the stack image for direct comparisons to the neurites they represent. The user interface is designed to facilitate manual inspection and correction of the reconstructed neuron to enhance reconstruction accuracy. The SWC output can then be analyzed to determine morphological differences between cells and to create computational models that enable the study of dendritic integration. As an open-source software tool, NeuTu will be used to create a database of reconstructed neurons that can be edited by individuals to improve the quality of the reconstructions and will give researchers access to high quality data on many different types of neurons.

P1592**Board Number: B103****MultiOmyxTM* immunocytochemistry reveals heterogeneous biomarker expression in human ESC and iPSC.**B. Davis¹, A. Larriera¹, M. Rothney¹, E. Kovacs¹, M. Gerdes¹;¹General Electric, Niskayuna, NY

Stem cells hold considerable promise for regenerative medicine, however, questions regarding the equivalency or homogeneity of different cell lines constantly arise, and this is exacerbated when iPSC lines are produced from somatic cells. It is well known that individual cell lines have a bias toward certain lineages through default differentiation pathways, and that cultures frequently undergo spontaneous differentiation. Monitoring the phenotype of the cells is complex, as no single marker defines the pluripotent state or lineage fate during directed differentiation. Likewise, directed differentiation protocols fail to fully induce all cells, with efficiencies of conversion to the desired phenotype being as low as 10% leading to heterogeneous mixtures of cells that ultimately need separation prior to applications in regenerative medicine such as transplantation. To address the heterogeneous nature of stem cell cultures and differentiated phenotypes, our team has applied

MultiOmyx™* immunocytochemistry to characterize these dynamic processes in human ESC and iPSC cultures in the pluripotent state as well as ES cultures undergoing directed differentiation to hepatocyte lineages. This approach allows sequential staining of the samples with intervening inactivation of dye conjugated primary antibodies and re-imaging on the same areas of the sample. We have successfully demonstrated 12 or more markers per sample and identified patterns of biomarker expression using algorithms developed for in situ single cell analysis within the colonies. For studies on ES and iPSC cells, traditional markers for pluripotency were examined including Sox2, SSEA-1, Nanog, Oct4, and ABCG2, revealing heterogeneous patterns of cellular localization within colonies undergoing phenotypic drift. In a separate study, efficacy of hepatocyte differentiation from the ES state was assessed by staining cultures for SOX17, FOXA2, OCT4, SOX2, BRACHYURY, Nestin and AFP, allowing process efficiency to be monitored. Use of MultiOmyx™* technology can characterize subpopulations within cultures, providing additional spatial and temporal details lost in conventional flow cytometry and immunocytochemistry methods.

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P1593

Board Number: B104

Stimulated emission depletion (STED) microscopy reveals a sub-diffraction, 9-fold symmetric domain containing Cby1, Ahi1, and Ofd1 at the centriole-cilium interface.

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Mutations and deletions of certain centriolar proteins result in a family of related syndromes called ciliopathies. For instance, deletion of the protein Cby1 results in cystic kidneys, a common ciliopathy phenotype. The centrioles' small size (~200 x 500 nm) is near the diffraction limit of light microscopy (~250 nm), making it difficult to precisely localize centriolar proteins, to determine their location with respect to other centriole proteins, and thus to build a molecular understanding of the detailed architecture of the centriole. We used stimulated emission depletion (STED) immunofluorescence microscopy with a FWHM resolution of 60 nm to image five centriolar proteins in multiciliated mouse tracheal epithelial cells (MTECs): Ahi1, Cby1, Ofd1, Sdccag8, and Tmem237 and find 9-fold symmetric structures. By calculating the radial intensity profile, the average radial positions of the five proteins were determined to better than 2 nm precision. This analysis revealed that Cby1 localizes near the distal centriole protein Ofd1 and the transition zone component Ahi1. Our work establishes a ring-shaped domain between the distal end of the centriole and the base of the cilium containing Cby1, Ahi1, and Ofd1.

P1594**Board Number: B105****Molecular Counting by Photobleaching in Protein Complexes with Many Subunits: Best Practices and Application to the Cellulose Synthesis Complex.**Y. Chen^{1,2}, N.C. Deffenbaugh¹, C.T. Anderson^{2,3}, W.O. Hancock^{1,4};¹Department of Biomedical Engineering, Pennsylvania State University, University Park, PA, ²Cell and Developmental Biology, Pennsylvania State University, University Park, PA, ³Department of Biology, Pennsylvania State University, University Park, PA, ⁴Interdisciplinary Graduate Degree Program in Bioengineering, Pennsylvania State University, University Park, PA

The constituents of large multi-subunit protein complexes dictate their functions in cells, but determining their precise molecular makeup *in vivo* is challenging. One example of such a complex is the cellulose synthesis complex (CSC), which in plants synthesizes cellulose, the most abundant biopolymer on Earth. In growing plant cells, CSCs exist in the plasma membrane as six-lobed rosettes that contain at least three different cellulose synthase (CESA) isoforms, but the number and stoichiometry of CESAs in each CSC is unknown. To begin to address this question, we performed quantitative photobleaching of GFP-tagged AtCESA3-containing particles in living *Arabidopsis thaliana* cells using variable-angle epifluorescence microscopy and developed a set of information-based step-detection procedures to estimate the numbers of GFP molecules in each particle. The step detection algorithms account for changes in signal variance due to changing numbers of fluorophores, and the subsequent analysis avoids common problems associated with fitting multiple Gaussian functions to binned histogram data. The analysis indicates that at least ten GFP-AtCESA3 molecules can exist in each particle. These procedures can be applied to photobleaching data for any protein complex with large numbers of fluorescently tagged subunits, providing a new analytical tool with which to probe complex composition and stoichiometry.

P1595**Board Number: B106****The use of time-lapse ptychography to measure angiogenesis assay dynamics.**R. Suman¹, K. Langley², K. Riches³, K.E. Porter³, P. O'Toole¹;¹University of York, York, England, ²Phasefocus, Sheffield, United Kingdom, ³University of Leeds, Leeds, United Kingdom

Endothelial cells (EC) play an important role in the formation of new blood vessels through angiogenesis. This functional capacity is conventionally analysed using angiogenesis assays whereby EC are plated onto a Matrigel substrate where they form tubes. The number of tubes formed are then analysed at fixed time points, e.g. 12 hours after seeding the cells. Using this fixed point method it is possible to identify treatments or disease states that modulate angiogenic capacity. Here we demonstrate the use of a ptychographic microscope (Phasefocus VL21) to study the dynamics of the angiogenesis/tube

forming assay through time lapse microscopy, negating the need for a fixed point assay that may miss key events and thus not reveal the whole biological dynamics of the tube formation process.

Ptychography is a novel imaging modality that produces high contrast label-free images of living cells. The technique reconstructs an image from overlapping diffraction patterns using an iterative algorithm (Maiden et al., 2010), generating both amplitude and phase data from the sample to report on quantitative changes in the refractive index (RI) and thickness of the specimen.

Large fields of view (2.4mm x 2.4mm, pixel size = 0.653 μ m) of human primary EC plated on Matrigel were imaged on the Phasefocus VL21 microscope every 10 minutes for periods up to one week. Quantitative parameters of the angiogenesis assay such as tube number, tube area and tube wall width were measured over time. With the availability of this temporal information we were able to identify dynamic changes in tube assembly and/or degradation. For example we were able to differentiate between tubes that failed to form and those that formed and subsequently broke down. This temporal data is not available using a fixed point assay where only the reduction in tube number would be evident.

In summary, we believe that this technique could form a new assay that provides additional information and reveal previously unavailable biological data to enable a more detailed understanding of the underlying mechanisms behind angiogenesis.

P1596

Board Number: B107

Multicolor correlative light and electron microscopy of single molecules: A case study of acetylcholine receptor localization at the mouse neuromuscular junction.

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With state-of-the-art on-section immuno-labeling proteins exposed to the surface of ultrathin sections can be labeled. However, using this technique not all existing epitopes are detected and resulting micrographs have to be evaluated carefully. To validate the on-section immuno-labeling of acetylcholine receptors (AChR) of the mouse neuromuscular junction (NMJ) on ultrathin sections and to study the AChR distribution we used a combination of different light and electron microscopic techniques. For registration of multi-modal images we established a fiducial-based workflow resulting in a robust and precise correlation better than 20 nm. By labeling AChR using fluorescent quantum dots (QDs) that can be detected in both imaging modes we are able to correlate single-molecular signals from both microscopes. First, the diaphragm muscle was explanted and AChR were labeled with α -bungarotoxin, a snake venom, conjugated to Alexa Fluor 555 (AF555). Subsequently the tissue was high pressure frozen, freeze substituted and embedded in Lowicryl resin. The AF555 fluorescence was preserved during this procedure giving the opportunity to study the whole resulting block using confocal laser scanning

microscopy to locate the structure of interest within the huge sample volume. Second, ultrathin sections were made and deposited either on indium tin oxide coated cover slips or on carbon coated grids with a formvar-film. The first type of samples enables subsequent super-resolution fluorescence light microscopy (FLM) based on multi-emitter localization microscopy and scanning electron microscopy. Analysis of a set of subsequent sections enables a three-dimensional representation of the preserved AF555 fluorescence which can be registered to the corresponding electron micrographs. Thereby the AChR distribution can be investigated in relation to the NMJ ultrastructure. The second type of samples is used for conventional FLM and for analytical scanning transmission electron microscopy (STEM). Additionally to the preserved AF555 fluorescence at AChR, receptors are labeled on-section using immuno-labeling with primary antibodies against a receptor subunit and secondary antibodies conjugated with two different QDs. QD fluorescence can be detected and distinguished in the FLM and using a novel workflow for the analytical STEM, particles can be separated according to their specific spectral features. By this means the distribution of multi-colored single molecules can be studied in light and subsequently in electron microscopy and resulting signals can be correlated. Combining these methods allows studying particular details by detection of single molecules within large volumes. As our workflows are modular they can be readily applied to other biological questions.

P1597

Board Number: B108

Shs1 is required for an organized septin higher order structure in vivo.

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Septins are conserved filament-forming proteins that act in cytokinesis, membrane remodeling, cell polarization, and migration. They closely associate with membranes and can act as diffusion barriers to restrict the diffusion of proteins within membranes. Although septin function is critical for diverse cell events, it is not well understood how they assemble *in vivo* or how they are remodeled throughout the cell cycle. GFP can be excited by and emit linear polarized light and linking GFP in a constrained manner to an endogenous septin enables analysis of septin organization *in vivo* by polarization microscopy. Polarized fluorescence analysis has previously suggested that septins filaments are paired in higher order structures, and that organized septin filaments undergo a coordinated 90° reorientation during cytokinesis *in vivo*. We developed a Multifocus Polarization Microscope (MF-PolScope) to evaluate septin organization in 3D through time to capture the assembly and rearrangement of higher-order septin structures, and analyze mutant yeast strains with abnormally organized septins. This method allows simultaneous detection of 9 focal planes at a single instant so as to analyze the degree of coordination of events in different areas of the cell and assess if filaments in mutant strains are likely paired. MF-PolScope imaging has led to the identification of septin interactors important for distinct aspects of the assembly, stability, and rearrangement of septins. One interactor necessary for ordered assembly of septin higher order structures is the septin, Shs1. The septin structure is disordered in *shs1*

mutant cells in a manner consistent with the absence of paired septin filaments. Our work demonstrates the power of this new imaging approach to assess dynamic rearrangements of the cytoskeleton in whole, live cells.

P1598

Board Number: B109

Nanoscale Analysis of Clathrin Coated Pit Formation using dSTORM.

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Clathrin mediated endocytosis (CME) is one of the major mechanisms of how cells retrieve membrane proteins and growth factors from the cell surface. CME depends on phosphoinositides (PIs) that regulate the recruitment and precise nanoscale organization of endocytic proteins (i.e. BAR proteins, PI-kinases, coat proteins). While initial stages of CME depend on PI(4,5)P₂ recent data have identified a crucial role for PI(3,4)P₂ in late steps of CME preceding dynamin-mediated fission (Posor et al. , 2013). The precise spatiotemporal organization of CME components is critical for the successful formation of clathrin coated pits (CCPs) that are diffraction limited structures of about 150 nm diameter. Here we use direct Stochastic Optical Reconstruction Microscopy (dSTORM) to investigate the nanoscale localization of the coat protein clathrin, the PI(3) kinase PI(3)KC2 α , the dynamin-associated PX-BAR domain protein sorting nexin 9 (SNX9) and the GTPase dynamin during CME. We observe distinct nanoscale distributions of these CME components in late stage CCPs. While clathrin and PI(3)KC2 α localize to the coat, dynamin is at the neck and SNX9 in an intermediate zone between coat and neck. Using live-cell imaging and electron microscopy we show that depleting SNX9 leads to lack of dynamin bursts at the end of CME and to the accumulation of U-shaped pits. Our data, supported by mathematical modeling, shows that PI(3,4)P₂ dependent recruitment of SNX9 to the neck-coat transition zone is necessary for successful CCP constriction and CME.

P1599**Board Number: B110****Nanoscale segregation of branched F-actin nucleation and elongation determines dendritic spine shape.**G. Giannone¹, A. Chazeau², A. Mehidi¹, D. Nair¹, J. Gautier³, C. Leduc⁴, F. Kage⁵, O. Thoumine¹, K. Rottner⁶, D. Choquet¹, A. Gautreau⁷, J. Sibarita⁸;¹Interdisciplinary Institute for NeuroScience, CNRS/Univ Bordeaux, Bordeaux, France, ²Utrecht University, Utrecht, Netherlands, ³Ecole Normal Supérieur, Paris, France, ⁴Institut Pasteur, Paris, France, ⁵Division of Molecular Cell Biology, Technical University Braunschweig, Braunschweig, Germany, ⁶University of Bonn, Bonn, Germany, ⁷CNRS, Gif-sur-Yvette, France, ⁸Institut Magendie - Univ Bordeaux2, Bordeaux, France

Actin dynamics drive morphological remodeling of neuronal dendritic spines and changes in synaptic transmission. Yet, the spatiotemporal coordination of actin regulators in spines is unknown. Using single protein tracking and super-resolution imaging we revealed the nanoscale organization and dynamics of branched F-actin regulators in spines. Branched F-actin nucleation occurs at the PSD vicinity, while elongation occurs at the tip of finger-like protrusions. This spatial segregation differs from lamellipodia where both branched F-actin nucleation and elongation occur at protrusion tips. The PSD is a persistent confinement zone for IRSp53 and WAVE complex, activator of the Arp2/3 complex. In contrast, filament elongators like VASP and formin-like protein-2 move outwards from the PSD with protrusion tips. Accordingly, Arp2/3 branch points are immobile and surround the PSD. Arp2/3 and Rac1 GTPase converge to the PSD, respectively, by cytosolic and membrane free-diffusion. Enhanced Rac1 activation and Shank3 over-expression, both associated with spine enlargement, induce delocalization of WAVE from the PSD. Thus the specific localization of branched F-actin regulators in spines might be reorganized during changes in synaptic activity to regulate spine structural plasticity.

P1600**Board Number: B111****Novel spectral imaging and analysis to unravel the organelle interactome.**A.M. Valm¹, S. Cohen¹, W.R. Legant², M.W. Davidson³, E. Betzig², J. Lippincott-Schwartz^{4,5};¹Cell Biology and Metabolism Program, Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH, Bethesda, MD, ²HHMI/Janelia Farm Research Campus, Ashburn, VA, ³National High Magnetic Field Laboratory and Department of Biological Science, Florida State University, Tallahassee, FL, ⁴Physiology Course at Marine Biological Laboratory, Woods Hole, MA, ⁵Cell Biology and Metabolism Program, NICHD, NIH, Bethesda, MD

The organization of the eukaryotic cell into membrane-bound compartments allows for regulation of cellular processes. However, the morphodynamic activities of spatially distinct organelles must be coordinated so that cellular processes can be carried out. Inter-organelle membrane contacts have emerged as key sites for exchange of metabolites and signaling molecules, as well as playing roles in

organelle division and maturation. Much progress has been made recently in elucidating the functions of pairwise organelle interactions, including the interaction between mitochondria and the endoplasmic reticulum (ER). However, it is likely that important cellular activities are coordinated between three or more organelles over time. For example, lipid droplets have been shown to interact with multiple organelles, including the ER, mitochondria, peroxisomes and the endocytic system, presumably in order to facilitate exchange of lipids in response to changing metabolic needs. A systems-level understanding of the dynamic organelle interactions within eukaryotic cells, i.e., the organelle interactome, remains unattained due to the inability to label and distinguish more than a few different fluorescent proteins in a single sample. Here, we present a novel cell labeling, image acquisition and analysis approach to study the spatial distribution of multiple organelles within single eukaryotic cells. Cells are transfected with multiple fluorescent fusion proteins targeted to various organelles or with organelle-specific fluorescent chemical dyes to label 6 or more different subcellular compartments. Live-cell, time-lapse images are acquired of labeled cells using commercially available imaging systems, then linear unmixing algorithms are applied to the recorded image data to deconvolve spectrally overlapping fluorophores. A computational image analysis procedure is then used to track the observed inter-organelle contacts through time. We further adapted a lattice light sheet imaging system for spectral imaging by introducing multiple laser lines coupled with fast acousto-optical beam splitting to record spectral images in the excitation regime. We developed a novel excitation-side unmixing algorithm and applied it to the recorded data to reconstruct full isotropic three dimensional time lapse images of live cells labeled with 6 different organelle markers. We imaged cells that simultaneously express fluorescent fusion protein markers for peroxisomes, lysosomes, mitochondria, the ER and the Golgi apparatus, as well as a vital chemical stain to label lipid droplets. We applied our organelle interactome analysis to test the hypothesis that there are subpopulations of lipid droplets within mammalian cells that differ in their organelle interactions.

P1601

Board Number: B112

Learning to count: Calculating receptor densities on membranes from super resolution images.

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Quantifying the number of labeled biomolecules from super resolution images is difficult due to artifacts from both over-counting and under-counting. We use over-counting artifacts in super-resolution images to calculate receptor density in cells. This method takes advantage of the observation that the magnitude of self-clustering due to over-counting is inversely proportional to the density of the labeled protein, as long as the labeled protein is sampled randomly. Pair autocorrelation functions calculated from over-counted fluorescence images are correlated ($g(r) > 1$) at radii on the order of the resolution limit of the measurement. We also demonstrate the effectiveness of this technique by using single-color STORM imaging to estimate EGFR density under different conditions. This method faithfully

recapitulated previously reported numbers for EGFR per cell in MCF-7 cell line. Furthermore, the absolute density of EGFR in different cell lines measured using this method agreed with relative densities determined using quantitative Western blots. We have further extended this method to quantitate interactions between labeled proteins in single cells by using two-color super resolution imaging. Cross correlation between two color channels have been used earlier in various studies as a measure of the strength of interaction between labeled proteins. Here, we combine cross correlation measurements with the protein densities calculated from autocorrelation functions to determine the number of interacting proteins in a single cell. We illustrate how this method complements existing biochemical techniques by investigating how the interaction of EGFR with the heat shock protein, HSP90 or E3 ubiquitination enzyme, Smurf2 affects its stability. Upon EGF stimulation, the density of Smurf2 on membrane increased while that of EGFR decreased. siRNA knockdown of Smurf2 increased EGFR degradation from the membrane upon EGF treatment, suggesting that Smurf2 stabilized activated EGFR. In support of this, we observed increased the interaction between EGFR and Smurf2 upon EGF stimulation. These results along with our previous work on HSP90-EGFR interaction show that the stability of EGFR in the plasma membrane is enforced by toggling its interaction of EGFR with these two proteins. Monomeric EGFR is stabilized through its interaction with HSP90, while in its activated state, it gets stabilized through monoubiquitination through interaction with Smurf2. Super-resolution methods have been used successfully to dissect several biological problems and our method of determining protein densities will extend the application of super-resolution to complement western blots and co-immunoprecipitation techniques that have traditionally been used to study biochemical pathways.

P1602

Board Number: B113

Lattice light sheet microscopy: imaging molecules, cells, and embryos at high spatiotemporal resolution.

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By definition, living specimens are animate. Therefore, a full understanding of dynamic biological systems will only be obtained by observing them with enough 4D spatio-temporal resolution and for a sufficient duration, to capture the phenomena of interest. Unfortunately, conventional widefield or confocal microscopes are either too slow, lack the spatial resolution, or induce too much photodamage to meet these requirements. To address these limitations, we have developed a new approach for sub-cellular light-sheet microscopy capable of imaging fast three-dimensional (3D) dynamic processes *in vivo* at signal to noise levels typically obtained only in total internal reflection fluorescence (TIRF) illumination. By utilizing 2D optical lattices, we generate a thin (~400 nm full width half maximum) plane of light coincident with the focus of a high NA detection objective. Using this technique, we demonstrate substantial advantages in speed, sensitivity and reduced phototoxicity compared to conventional point scanning and spinning disc confocal microscopes as well as light-sheet microscopes

utilizing single Gaussian or Bessel beams. We leverage these advantages to image samples ranging over three orders of magnitude in length scale from single molecules to whole embryos. Specific examples to be presented include: 3D single molecule tracking of fluorescently tagged transcription factors in densely labeled embryonic stem cell spheroids, 3D imaging and quantification of microtubule growth phases and organelle dynamics throughout the course of cell division, rapid 3D imaging of Tetrahymena motility at over 3 volumes per second, and 3D protein localization throughout the course of dorsal closure in Drosophila embryos. By combining lattice light sheet microscopy with novel fluorescent probes, we will also demonstrate 3D super-resolution localization microscopy with unprecedentedly rich detail over large fields of view and in thick 3D samples such as dividing cells and small embryos.

Proteomics, Subcellular Localization, and Molecular Interaction Technologies

P1603

Board Number: B114

The Subcellular Protein Atlas.

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Mapping proteins to subcellular compartments is key for understanding their function and interactions, and further, the complex machinery of eukaryotic cells. Despite vast research, much is still unknown regarding the spatial organization and biological processes of many proteins.

The Subcellular Protein Atlas aims to systematically localize the entire human proteome on a subcellular level, using an antibody-based approach as part of the Human Protein Atlas project. In order to find suitable samples expressing the target protein, the panel of cell lines has been expanded and RNA-seq is used to cherry pick the best cell line for each protein (1). More than 10.000 human proteins have been localized and validation methods applied include gene silencing using siRNA for antibody binding and protein localization (2), fluorescently tagged proteins (3) and paired antibodies targeting the same protein.

Our results indicate that there exist distinct organelle proteomes that are common for cell lines, with a strong correlation to protein function. Also, our results suggest that approximately 50% of all proteins localize to multiple compartment, whereas 5-10% show a cell cycle dependent expression pattern (4). In addition, in order to localize the full human proteome, our data imply that cells of different origin will need to be used, pointing towards cells with hematopoietical or neurological functions in complement to adherent epithelial cells. We have thus optimized our high-throughput protocols to enable characterization of cells in suspension, stem cells and differentiated neurons.

Here we discuss the importance of spatial proteomics and present the content and results of the Subcellular Protein Atlas. We also discuss the path to successfully analyze the full human proteome on a subcellular level.

Wiking, *et al*, (2013), *J Proteome Res*

Stadler, *et al*, (2012), *J Proteomics*

Stadler, *et al*, (2013), *Nat Methods*

Stadler, *et al*, (2011), *J Proteome Res*

P1604

Board Number: B115

Novel surface functionalized superparamagnetic nanoparticles with engineered cellular uptake as a means for intracellular omics.

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Superparamagnetic nanoparticles (SPMNPs) are appealing for use in organelle isolation strategies. Yet, this potential remains largely unexplored because thus far research has focused on either physicochemical design or on their application in micron-sized beads. For their use in life sciences, the biocompatibility of SPMNPs goes beyond their chemical composition and shape, and features like their size and more importantly their surface properties are becoming more important to exploit extra- and intracellular interactions. Here we introduce thermal decomposition to manufacture iron oxide based SPMNPs (Ø10nm) and demonstrate how different surface functionalizations can lead to different types of cellular interactions. Cationic aminolipid-coated SPMNPs reside surprisingly strong at the outer cell surface. In contrast, anionic dimercaptosuccinic acid-coated SPMNPs are efficiently internalized and accumulate in a time-dependent manner in endosomal and lysosomal populations. These features allowed us to establish a standardized magnetic isolation procedure to selectively isolate plasma membranes and intracellular late endosomes/lysosomes with high yields and purities as consolidated by biochemical and ultrastructural analyses. Subsequent quantitative and qualitative proteome analysis

underpins the overall high enrichment for hydrophobic (membrane) proteins as well as plasma membrane and lysosomal constituents in the respective purified fractions. This nano based technology provides therefore a breakthrough in the field of subcellular 'omics' as it allows the identification of subtle alterations in the biomolecular composition of different SPMNP-isolated compartments that would be otherwise not detected in total cell or tissue analysis.

P1605

Board Number: B116

Developing Mitochondrial Enrichment for Targeted Analysis (META) to Study Phosphorylation Kinetics.

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Mitochondria are a dynamic organelle whose localization, morphology and function are subjected to precise regulation. It is known mitochondria activity differs vastly in different tissues. Investigating the post-translational modifications of mitochondrial proteins is crucial to understand mitochondria activity under both physiological and pathological conditions. The main challenge in this kind of studies is the fact that the isolation procedures used to prepare purified/enriched mitochondria severely affects post-translational modifications by triggering, for example, massive dephosphorylation and proteolysis. To overcome this issue, we developed Mitochondria Enrichment for Targeted Analysis (META), a streamlined workflow to study changes in protein phosphorylation profile in mitochondria isolated from mouse salivary glands. This two-step approach includes mitochondrial enrichment with minimal tissue disruption and analysis via targeted mass spectrometry using a library of putative phosphorylation sites in mitochondrial proteins. For sample preparation, cryofixation and subsequent sectioning were performed to preserve mouse salivary glands. We probed mitochondria in the prepared tissue samples with mitochondria-specific antibody anti-TOM20 and applied expression-based microdissection (xMD) to isolate mitochondria from the tissue onto polymer films. The films containing dissected mitochondria were then subjected to DAPI staining and Scanning Electron Microscopy (SEM) to visually verify the exclusion of nuclei and presence of mitochondria. The efficiency of microdissection was assessed to be 45.91% by comparing the segmented images of the tissue before and after xMD. Following protein extraction we evaluated the extent of mitochondrial protein enrichment by immunoblot assay and found cytochrom C to beta-actin ratio to be 152% higher compared to the manual microdissection controls. In order to systematically study the dynamic changes of phosphorylation in response to stimuli in mitochondrial proteins using targeted mass spectrometry, putative phosphorylation sites of mitochondrial proteins were predicted *in silico* and compiled into a library, where a total of 231 proteins and 42,966 possible M/Z peak changes are listed. In summary, our results show that META is a powerful tool for mitochondria enrichment from tissue and guiding subsequent proteomics profiling. Moreover, this workflow can also be applied to study protein dynamics in mitochondria in other tissue types as well as to characterize protein dynamics in other organelles.

P1606**Board Number: B117****ENGINEERED CONTROL OF PROTEIN TYROSINE PHOSPHATASE ACTIVITY IN LIVING CELLS.**J.E. Brinks Klomp¹, V. Huyot², A.V. Karginov¹;¹Pharmacology, University of Illinois - Chicago, Chicago, IL, ²Pharmacology, University of Illinois at Chicago, Chicago, IL

We describe here a new broadly applicable approach to activate the catalytic function of specific protein phosphatases in living cells. Insertion of an engineered allosteric switch, the iFKBP domain, at a structurally conserved position within the catalytic domain makes the modified phosphatase inactive. Treatment with rapamycin triggers interaction with a small FKBP-rapamycin-binding domain (FRB) and restores the activity of the phosphatase. The method utilizes genetically encoded and membrane permeable reagents providing tight temporal control of phosphatase activity. Based on the structural similarity of catalytic domains this method should be applicable to many tyrosine phosphatases. We have already developed rapamycin-regulated (RapR) analogs of Shp2, PTP-PEST and PTP1B. Using PTP-PEST as an example we demonstrate that amino acid sequence alignment provides sufficient information for generation of new RapR-phosphatases. Through conjugation of FRB to a desired protein, we are able to restrict phosphatase activation to interaction with a specific downstream target, and to specific subcellular locations. This approach opens new opportunities for interrogation of phosphatase-mediated signaling pathways.

P1607**Board Number: B118****Insertion of LOV and engineered domains to control kinases, Rho GTPases and GEFs with light or small molecules.**O. Dagliyan¹, P. Chu², M. Tarnawski³, M. Shobair¹, D. Shirvanyants¹, I. Schlichting³, N. Dokholyan¹, K.M. Hahn²;¹Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC, ²Department of Pharmacology, University of North Carolina, Chapel Hill, NC, ³Department of Biomolecular Mechanisms, Max Planck Institute for Medical Research, Heidelberg, Germany

For many cellular behaviors, it is essential that signaling is precisely coordinated in space and time. Such spatiotemporal dynamics can only be fully understood using tools that examine and manipulate protein behavior in intact living cells. Here we report the insertion of engineered domains to inhibit kinases, GTPases and guanine exchange factors (GEFs) with light, and activate GEFs with small molecules. Previously, we developed an approach in which a ligand-controlled engineered domain was inserted into the surface loops of kinases, where it exerted allosteric control of kinase activity. Kinases were rendered catalytically inactive until rapamycin analogs rescued activity. By building switchable Src family kinase

isoforms, including RapR Src, Fyn, and Lyn, we were able to demonstrate different roles and localization for each isoform at specific stages of polarization. Here, we show the approach can be applied to a different protein family, Rho GEFs, which are activators of small GTPases. Molecular dynamics and other approaches indicated allosteric interactions between active sites and distant residues, guiding insertion of an engineered “RapR domain” into Vav2 and intersectin, conferring rapamycin-induced activation. By inserting the light sensitive LOV domain, we produce GEFs, GTPases and kinases inhibited by light. We will describe ongoing studies focused on protein interactions mediating different stages of cell polarization.

P1608

Board Number: B119

A statistical framework to analyze molecule colocalization.

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The quantitative analysis of molecule interactions in bioimaging is key for understanding the molecular orchestration of cellular processes and is generally achieved through the study of the spatial colocalization between different populations of molecules.

Most colocalization methods are based on pixel overlap between the previously denoised signal that is emitted from two (or more) different fluorescent labels, and use a global image correlation such as Pearson's or Manders' coefficients. These data, however, cannot be linked to physical parameters such as the real percentage of colocalizing molecules or the average colocalization distance. In addition, randomly distributed molecules can partially overlap, and it is hard to measure the statistical significance of the computed correlation indices. Here, we present a novel statistical method to analyze molecule colocalization that is based on the automatic detection of molecule fluorescent spots, followed by their representation as Point Processes and the statistical analysis of their spatial distribution. This allows to :

1. Test robustly and rapidly whether two molecule distributions colocalize in space, thanks to a closed-form analysis with no need for simulations;
2. Measure statistically the real percentage of colocalizing molecules and the interaction mean distance. Using our method and Total Internal Reflexion Fluorescence (TIRF) microscopy, we first analyse the spatial colocalization between different endocytic cargos and cellular molecules at the cell membrane. Moreover, by coupling our statistical method to single-particle tracking techniques, we propose an automated framework to study the dynamical interactions of molecules and the endocytosis orchestration.

P1609**Board Number: B120****A new variant of BioID to identify protein-protein associations in live cells.**D. Kim¹, S.C. Jensen¹, K. Noble², R.H. Anderson¹, B. KC¹, K. Motamedchaboki³, K.J. Roux¹;¹Children's Health Research Center, Sanford Research, Sioux Falls, SD, ²Department of Biology, Florida State University, Tallahassee, FL, ³Sanford-Burnham Medical Research Institute, La Jolla, CA

Identification of protein-protein interactions is frequently a critical facet to the process of biological discovery. To address technical barriers to monitoring protein-protein associations in living cells we developed proximity-dependent biotin identification (BioID). This method is based on the cellular expression of a promiscuous biotin ligase fused to a protein of interest, thus generating biotinylation of endogenous proteins in a proximity-dependent manner. These biotinylated proteins can be identified by mass-spectrometry following biotin-affinity capture. Although BioID has rapidly become an established method to monitor protein interactions, there remain limitations in its application. One constraint results from the size of the promiscuous biotin ligase used for BioID, which is approximately 35kDa. We have found that certain integral membrane proteins are unable to target appropriately when fused to BioID. To overcome this limitation, we have developed a substantially smaller promiscuous biotin ligase called BioID-2 (approximately 27 kDa). BioID-2 permitted appropriate targeting of fusion proteins that were improperly targeted with BioID. We applied both BioID and BioID-2 to LaA, a major constituent of the nuclear lamina and detected similar candidates thus revealing that BioID-2 is functionally comparable to BioID. Comparative studies of BioID and BioID-2, both in vivo and in vitro, reveal differences in enzyme properties that may indicate preferential applications for each variant. Collectively, these studies expand the capabilities of the BioID method.

P1610**Board Number: B121****Probing the nuclear pore complex with BioID.**D. Kim¹, B. KC¹, K. Motamedchaboki², V. Doye³, K.J. Roux¹;¹Children's Health Research Center, Sanford Research, Sioux Falls, SD, ²Sanford-Burnham Medical Research Institute, La Jolla, CA, ³Inst Jacques Monod-UMR 7592 CNRS-Univ Paris Diderot, Paris Cedex 13, France

Proximity-dependent biotinylation (BioID) is a method to identify protein associations that occur in living cells. Fusion of a promiscuous biotin ligase to a bait protein for cellular expression enables covalent biotin labeling, selective isolation, and identification of proteins proximate to the bait. This effectively generates a history of protein associations that occurred during a defined labeling period in live cells. We have used BioID to study the human nuclear pore complex (NPC), a large macromolecular assembly. BioID was applied to constituents of the Nup107-160 complex and the Nup93 complex, two conserved NPC subcomplexes. A strikingly different set of NPC constituents was detected depending on the position of the BioID-fusion proteins within the NPC. By applying BioID to multiple constituents located

throughout the extremely stable Nup107-160 subcomplex we have refined our understanding of this highly conserved subcomplex, in part by demonstrating a direct interaction of Nup85 with Nup43. And by using the extremely stable Nup107-160 structure as a molecular ruler we have defined the practical labeling radius of BioID, thus enabling a more rational use of the method and interpretation of results. Furthermore, we have extended these studies to include characterization of a BioID enzyme variant. These studies further our understanding of human NPC organization and demonstrate that BioID is a valuable tool to explore the constituency and organization of large protein assemblies in living cells.

P1611

Board Number: B122

Localized light-induced protein dimerization in living cells using a photocaged dimerizer.

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Regulated protein localization is critical for many cellular processes. We present a new technique to rapidly and reversibly control protein localization in living human cells with subcellular spatial resolution using a cell permeable, photoactivatable chemical inducer of dimerization. This novel dimerizer consists of a Halotag ligand linked to a photocaged derivative of trimethoprim (TMP). The Halotag ligand covalently binds to Halotag fusion proteins, which serve as anchor domains for protein relocation experiments. Photocaged TMP remains inert until illuminated with ~400 nm light, at which point the unmasked TMP non-covalently binds *E. coli* dihydrofolate reductase (EcDHFR), thereby dimerizing EcDHFR and Halotag fusion proteins. Using this system, we demonstrate light-induced recruitment of a cytosolic protein to individual centromeres, kinetochores, mitochondria, and centrosomes in living human cells. Several systems for light-induced dimerization utilizing naturally light-sensitive proteins have been developed, but none of these systems have been successfully applied at centromeres or kinetochores, complex chromatin structures which are crucial for cell division. Although the photochemical uncaging reaction is irreversible, dimerization can be reversed by addition of free TMP. This work represents a new strategy for controlling protein localization with light using a modular photocaged dimerizer covalently pre-localized to an anchoring protein. This technique should be readily and widely applicable at a variety of cellular locations to address a range of biological questions.

P1612**Board Number: B123****Fluorescence-based, live-cell assessment of ER stress.**M. Liljevald¹, D. Swahn¹, U. Andersson¹, V.M. Loitto²;¹Drug Safety and Metabolism, AstraZeneca RD, Molndal, Sweden, ²Medical Microbiology, Dept. of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden

Cell-based, *in vitro* alternatives to toxicity testing are very much needed to reduce the number of laboratory animals. We are working on setting up a set of cellular assays composed of cell- and organelle-specific analyses, such as mitochondria, membrane integrity, cell cycle, and endoplasmic reticulum. An important part of this battery of assays is assessing cellular stress, more specifically the unfolded protein response. As an initial effort we are currently testing the IRE1a/XBP1 pathway for which we have designed novel fluorescent probes. For this purpose, we have made probes that are FRET-based and probes that have stress-inducible onset of fluorescence. With both conventional expression plasmids as well as lentivirally generated stable cell clones, we are currently validating the novel probes. In initial experiments using HepG2 cells, we have found that probes assessing IRE1a activation of the XBP1 pathway accurately and dose-dependently reflect exposure to known ER stress inducers such as Tunicamycin and Brefeldin A and other compounds that are associated with liver toxicity in the clinic. Using the fluorescence-inducing XBP1-probe, cells treated with 2 μ M Tunicamycin or 50 nM Brefeldin A for 24h shows 10-15 times increased fluorescence compared to controls. We have also identified compounds that inhibit ER-stress responses and may act as molecular chaperones, which could be of potential therapeutic benefit in several diseases where ER stress plays important role such as NASH and diabetes. Our long-term goal is to reduce the number of animals needed for ranking novel drug candidates and to reliably predict possible adverse effects.

P1613**Board Number: B124****A Novel Fluorophore-Based Imaging Method Is Useful for Studying the Substrate Specificity of Gut Hydrolases in *Caenorhabditis elegans*.**S. Dube¹, H. Dube¹, N.B. Green^{1,2}, R.J. Johnson², J.R. Kowalski¹;¹Biological Sciences, Butler University, Indianapolis, IN, ²Chemistry, Butler University, Indianapolis, IN

Traditionally, the expression and localization of proteins has been studied *in vivo* by fluorescent tagging. The first such experiments with green fluorescent protein (GFP) were conducted in *Caenorhabditis elegans*, as these transparent roundworms are amenable to genetic manipulation and fluorescence microscopy. With one fluorescent tag per protein, however, this method cannot quantify subtle changes in protein levels, such as those due to the action of regulatory mechanisms. Our goal was to develop a more sensitive *in vivo* method to amplify the fluorescent signal and allow for rapid, cell type-specific quantification of small changes in protein abundance. We chose to focus on neuronal proteins since their abundance and activity are tightly regulated and because *C. elegans* shares conservation of

neuronal proteins and nervous system structure with humans. We made use of an enzyme-activated fluorescence system recently developed by Tian *et al.* (2012), in which they synthesized an ester-masked version of fluorescein, the fluorescence of which could be activated in a cell-specific manner by the orthologous esterase, porcine liver esterase (PLE), when expressed in cultured mammalian cells. We attempted to apply this method *in vivo* by delivering ester-masked fluorophores to *C. elegans* with neuronal expression of PLE. Since fluorophore delivery is hindered by the cuticle, a proteinaceous covering around the worms, we synthesized two ester-masked fluorescein derivatives with hydrophobic tails of either 12 or 18 carbons in length to aid uptake into the plasma membranes of externally exposed sensory neurons. We confirmed *in vitro* activation of these masked fluorophores by purified recombinant PLE. We also created transgenic *C. elegans* strains with neuron-specific PLE expression, which we verified by RT-PCR and Western blot. Though we have not seen neuronal activation of our ester-masked fluorophores in the transgenic worms, we have observed fluorescence activation in gut granules of both wild-type and PLE-expressing animals. Thus, we now believe this esterase-ester-masked fluorophore system will be useful for identifying and characterizing the substrate specificities of gut hydrolases in *C. elegans*, an underexplored area of research in this otherwise well-characterized animal. We are currently delivering masked fluorescein derivatives to various mutant strains to determine the identity and specificity of these digestive enzymes.

P1614

Board Number: B125

Real-Time Observation of Lipid-Protein Interactions in Crude Cell Lysates with Single-Molecule Resolution.

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Lipid-protein interactions play key roles in signal transduction. Obtaining mechanistic insights of these interactions is obligatory for a better understanding of biological processes. Here we have extended a single-molecule pull-down assay (SiMPull) to probe lipid-protein interactions in crude cell lysates. Single-molecule data analysis is performed to quantitatively describe the assembly of lipid-binding domains on their target lipids. Importantly, this assay is applicable to full-length proteins expressed in crude cell lysates, as shown for the protein kinase AKT which binds specifically to the lipid PI(3,4,5)P₃. Our results demonstrate proof-of-principle for the application of this new assay to investigating protein-lipid second messenger interactions at high resolution in whole cell lysates, eliminating the need of harsh and lengthy procedures employed in biochemical purification.

P1615**Board Number: B126****A sentinel protein assay for the simultaneous quantification of cellular processes.**M. Soste¹, R. Hrabakova¹, S. Wanka², A. Melnik¹, P. Boersema¹, C. von Mering², P. Picotti¹;¹Institute of Biochemistry, ETH Zürich, Zurich, Switzerland, ²Institute of Molecular Life Sciences, University of Zürich, Zurich, Switzerland

Here we introduce a novel proteomic screening approach that provides a system-wide, quantitative snapshot of the activity status of a variety of cellular processes, simultaneously. The approach is based on the concept of sentinel proteins. Sentinels are biological markers whose change in abundance characterizes the activation state of a given pathway or functional module in a cell. Sentinels are proteins, phosphorylation sites or protein degradation products that have been previously validated and are commonly assayed in molecular biology laboratories, typically one at a time by traditional antibody-based techniques. For example, sentinels include: specific phosphorylation events in the activation loop of MAP kinases as markers for signalling along different MAPK branches; or induction of the protein Atg8 (LC3 in mammals) as a marker for the activation of the autophagic response. By combining previous biochemical knowledge and computational prediction, we selected a set of 309 sentinels to probe the physiology of *S. cerevisiae* cells. We assembled an information-rich targeted proteomic fingerprint assay based on selected reaction monitoring-mass spectrometry (SRM-MS) that measures the complete sentinel set at high-throughput (1 hour per sample), and reports on the activation state of 188 biological processes. To validate the approach, we applied it to a set of eight well-characterized environmental perturbations of yeast physiology. We show that it recapitulates many known responses and enables the identification of novel cellular events, thereby demonstrating that the sentinel approach could be used to analyse the cellular responses to a large set of uncharacterized perturbations, such as a collection of drugs.

Our current interest is in applying the approach to characterize at high-throughput the modulation of cytotoxicity induced by the aggregation-prone protein, alpha-synuclein. Using yeast as a model system, cytotoxicity induced by alpha-synuclein has been shown to be dose- and time-dependent yet can be modulated by the co-overexpression of ~100 different protective genes. We are using the sentinel assay to decipher the effects of these mostly uncharacterized genetic modulators of toxicity. By screening the activity status of biological processes in yeast rescued by these modulators, we should be able to identify effectors of the rescue. The findings of this study should highlight the processes most crucial to rescue from alpha-synuclein toxicity and provide targets for follow-up in higher models.

P1616**Board Number: B127****Near Infrared (NIR) Reversible Total Protein Staining as an Alternative Loading Control Method for Quantitative Fluorescent Western Blotting.**R. Skopp¹, S. Day², T. Urlacher², N. Padhye²;¹LI-COR Inc., Lincoln, NE, ²LI-COR Biosciences, Lincoln, NE

In Western blotting, a suitable internal protein loading control is essential for accurate and quantitative comparisons of various sample types. Selecting a proper loading control is critical to ensure reliable evaluation of protein amounts across various samples, to reduce errors introduced by sampling irregularities and uneven protein transfer across a membrane. Traditionally, housekeeping genes such as tubulin, actin and GAPDH have been used to normalize protein loading as they were thought to be expressed at consistent levels across nearly all tissue types and experimental conditions. However, recent studies have found that the expression and therefore protein concentration of housekeeping genes may fluctuate depending on expression conditions and treatments. Unbiased detection of all proteins in each sample lane would provide a more versatile and robust method for normalizing protein loading amounts in Western Blots. Traditional methods of normalization using total protein analysis require samples to be run in parallel where one set is stained using visible stains; the other set is used for immunodetection. Visible protein stains such as Ponceau S, Amido Black and Coomassie blue have several limitations including long incubations, difficult preparation and irreversible protein staining; they also lack the sensitivity required for normalization. Here we present a simple, rapid, sensitive and reversible method for staining total protein on a membrane using Direct Blue 71 (DB 71). The stain provides visual confirmation of transfer of proteins to an immobilizing membrane. Further, the fluorescence signal can be used to quantify the amount of protein in individual lanes or bands. After quantitation, the stain can be removed from the membrane without interference or disruption of downstream processing by Western blot. For quantitative Western analysis, a correlation between the results and the protein amounts in samples must be established. DB 71 staining exhibits sensitive linear response from 1 to 40 μg of cell lysate, with r^2 values of 0.97. The normalization of proteins in stimulated and unstimulated cell lysates using DB 71 total protein staining was comparable to normalization using housekeeping genes (Erk and Actin). Thus, DB 71 staining can be used as a robust alternative loading control method for NIR quantitative fluorescent Western blotting with advantages which include ease of use, wide linear range, quantitative accuracy, reversibility and compatibility with immunodetection.

P1617**Board Number: B128****Large-scale analysis of the evolutionary history of phosphorylation motifs.**S. Okuda¹, H. Yoshizaki²;¹Niigata University, Niigata, Japan, ²Kanazawa Medical University, Kanazawa, Japan

Protein phosphorylation is catalyzed by kinases and is involved in the regulation of a wide range of processes. The phosphosites in protein sequence motifs determine the types of kinases involved. The development of phosphoproteomics has allowed the identification of huge numbers of phosphosites. Employing the known phosphosite data enabled us to develop a novel method detecting protein phosphorylation related to important cellular functions. Known phosphosites retrieved from the large-scale database were mapped to the human genome and the peripheral regions including the phosphosites were extracted. Subsequently, we clustered the sequences by the homology and created our original phosphomotifs. To trace the evolutionary transition of the three amino acid residues, serine, threonine, and tyrosine, of the phosphosites, we performed a comparative genome analysis and investigated the conservations from yeast to human. We observed that several phosphomotifs identified by our method were frequently acquired at a certain point in an evolutionary scenario. We also showed that the motifs present in specific species functioned in an additional network that interacted directly with the core signaling network conserved from yeast to humans. Our method may facilitate the efficient extraction of novel phosphomotifs with physiological functions, thereby contributing greatly to the analysis of complex phosphorylation signaling cascades. Our study suggests that the phosphorylation networks acquired during evolution have added signaling network modules to the core signaling networks.

P1618**Board Number: B129****Comprehensive Phenotyping and Functional Profiling of the Immune System with Single Cell Resolution using Mass Cytometry.**M. Poulin¹, M. Konrad¹, S. Han¹, L. Chokhani¹, T. George¹, P. Delucchi¹;¹Fluidigm Sciences Inc., South San Francisco, CA

Understanding the heterogeneity inherent to normal and diseased tissue requires multi-parametric analysis with single-cell resolution. Mass cytometry is a powerful new single-cell proteomics platform that enables analysis of at least 30 proteins per cell simultaneously, and at high cellular acquisition rate, without the requirement for compensation. Antibodies directed against protein targets are labeled with isotopically pure rare earth metals and quantified by inductively coupled plasma mass spectrometry (ICP-MS). Single cell analysis of the proteome by mass cytometry enables rich phenotypic sub-categorization of cells and reveals, within these populations, different functional states defined by cell activation status, cell cycle progression and effector protein expression. In this study, panels of both human- and mouse-specific metal conjugated antibodies against surface markers were used to

phenotype all of the major immune cell subsets. These surface panels were then systematically combined with complementary antibody panels designed to characterize cell cycle status, signaling activity, or effector cytokine expression in each of the identified immune cell subsets. We demonstrate that mass cytometry enables an unprecedented ability to resolve the functional profile of the entire immune cell compartment.

P1619

Board Number: B130

Near Infrared Chemifluorescent Detection for Enhanced Western Blot Sensitivity.

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¹LI-COR Biosciences, Lincoln, NE

Detection of low-abundance proteins in a complex mixture is a key requirement for biotechnology research. Here we describe a method for highly sensitive protein detection on Western blots using a horse radish peroxidase (HRP) substrate with excitation and emission at near infrared wavelengths. Film detection of HRP mediated reactions has long been the standard for Western blot detection. Digital imaging has slowly supplanted film detection due to increasing film and hazardous waste expenses, but some targets are only detectable using film with high sensitivity chemiluminescent substrates. Detection of HRP reactions in the NIR has several advantages over chemiluminescent detection, such as extending the dynamic range compared to film by using digital imaging. The chemifluorescent reaction produces a stable fluorescent signal detected at 700nm which can be quantified to determine the linear range. We demonstrate up to 8-fold improved sensitivity with NIR chemifluorescent detection of a low-abundance phosphorylated protein and hormone receptor compared to the highest sensitivity chemiluminescent substrate. The low relative abundance of many proteins within a given mixture and the limited quantity of precious sample make sensitive detection of target proteins highly desirable in Western blotting. We expect other protein detection methods such as ELISAs and protein arrays will also benefit from improved sensitivity.

P1620

Board Number: B131

Accurate absolute quantitation of endogenous protein using Simple Western.

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¹ProteinSimple, San Jose, CA

Why do researchers use Western blots? Since the technique was developed thirty years ago it's been a trusted method for confirming the presence or absence of a protein but lacks the quantitative rigor for accurate measurement of the amount of protein present. The process requires many steps which each introduce variability. While portions of the technique have been automated to improve consistency,

there hasn't been a major leap in the technology to propel this method of protein analysis from qualitative to quantitative.

Simple Western is the modern evolution of traditional immunoassay techniques. Wes, the latest addition to the Simple Western platform, is an easy to use, fully automated system that removes the variability seen with traditional Westerns for more reproducible results run to run and between users. Researchers can now identify their protein and achieve reliable quantitation of their target proteins quickly and with very little sample.

To demonstrate the ability of Wes to accurately quantitate protein, we ran spiked GST-tagged AKT1 to act as a standard curve into Jurkat cell lysate. The samples were run on both Wes and a traditional Western blot and when the two methods were compared, Wes not only reduced the hands on time from 120 to 30 minutes and time to results from 2 days to 3 hours, but the reproducibility of the data (9.4%CV vs. 34.5%CV) provides a higher degree of confidence in the accuracy of the reported concentration of endogenous AKT1. Why do researchers use Western blots? Since the technique was developed thirty years ago it's been a trusted method for confirming the presence or absence of a protein but lacks the quantitative rigor for accurate measurement of the amount of protein present. The process requires many steps which each introduce variability. While portions of the technique have been automated to improve consistency, there hasn't been a major leap in the technology to propel this method of protein analysis from qualitative to quantitative.

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P1621**Board Number: B132****Loss-of-Function Genetic Screens to Find Genes Regulating Cell Responses and Identify Potential Drug Targets.**P. Diehl¹, D. Tedesco¹, K. Bonneau¹, M. Makhanov¹, D. Deng¹, K. Hyder¹, P. Sun², A.A. Chenchik¹;¹Cellecta, Inc., Mountain View, CA, ²The Scripps Research Institute, La Jolla, CA

Loss-of-function genetic screens using RNAi provide one of the few ways to identify genes essential for growth. With cultured cells and complex pools of viral-based shRNA expression libraries with constrained representation, these sort of screens can be efficiently and cost-effectively run on a genome-wide basis to identify genes essential for viability in the presence or absence of a compound or other factor. However, despite strong interest to use similar broad genetic screens with *in vivo* xenograft models to find genes essential for tumor growth, cell growth variability and low take rates create significant challenges in adapting the methodology to these systems. To address these challenges, we have designed novel pooled shRNA libraries that contain a unique barcode feature that enables assessment of each clonal cell population with a screen responds. This clonal tracking provides a more robust analysis of how each shRNA affects cellular growth within the tumor which significantly improves the reliability of the results from pooled shRNA genetic screens of xenografts.

Actin and Actin-Associated Proteins 3**P1622****Board Number: B134****Structural polymorphism of actin detected by intramolecular FRET *in vivo* and *in vitro*.**T. Noguchi¹, M. Okazaki¹, S. Kijima^{1,2,3}, M. Morimatsu⁴, A. . Nagasaki², Y. Iwadate⁵, T. Yanagida^{6,7}, T. Uyeda^{2,3};¹Dept. of Chem. Sci. and Eng., Miyakonojo Nat. Coll. of Tech., Miyakonojo, Miyazaki, Japan, ²Biomed. Res. Inst., Natl Inst Adv Indust Sci Technol, Tsukuba, Ibaraki, Japan, ³Grad. Sch. Sci., Univ. of Tsukuba, Tsukuba, Ibaraki, Japan, ⁴Chemical Engineering, Stanford University, Stanford, CA, ⁵Yamaguchi Univ., Yamaguchi, Yamaguchi, Japan, ⁶Osaka Univ, Suita, Osaka, Japan, ⁷QBiC, RIKEN, Suita, Osaka, Japan

Actin plays a variety of important roles in eukaryotic cells, and these functions depend on interactions with many actin-binding proteins (ABPs). Meanwhile, microscopic and biochemical studies suggested that actin takes multiple conformational states. This polymorphism of actin structure has been speculated to affect interactions with ABPs. However, such polymorphism of actin has not been detected in cells, and its physiological relevance is unclear.

To understand the effects of ABPs on actin structure, we developed actin with an intramolecular FRET probe, of which the two major domains (the small domain and large domain) were labeled with two

fluorescence dyes. The actin with the FRET probe was copolymerized with unlabeled actin, and the co-filaments were immobilized on a glass surface by the avidin-biotin system. Single-molecule observation of the actin protomer with the FRET probe in co-filaments revealed that actin subunits take at least two distinct structural states. Furthermore, addition of skeletal heavy meromyosin (HMM) and ATP changed the distribution of the actin structures. We also prepared G146V mutant actin with the FRET probe, since G146V mutant actin showed severe motility defect with skeletal HMM (Noguchi et al., 2012). Interestingly, the distribution of structures of the G146V actin protomer with the FRET probe was different from that of the control, suggesting that the motor activity of skeletal HMM needs optimal conformational changes of actin. Next, the actin with the FRET probe was introduced into PtK2 cells using our laboratory-built electroporator optimized for adherent cells, and were observed by confocal microscopy. The FRET ratio (intensities of acceptor/donor) in PtK2 cells differed locally, indicating that actin structure in cells is also variable. We speculate that such polymorphism of actin *in vivo* is correlated with subcellular localization of ABPs. We previously showed that myosin and cofilin each binds to actin filaments cooperatively *in vitro*. The two cooperative bindings are mutually exclusive, and we speculated that myosin and cofilin each stabilizes different conformations of actin filaments, and prefers to bind to the filaments with the tailored conformation (Kijima et al., submitted). We suggest that the polymorphism of actin we observed *in vivo* is correlated with differential affinities for various ABPs, and play important roles in subcellular localization of the ABPs.

P1623

Board Number: B135

Structural basis of Arp2/3 Complex Inactivation revealed by single particle EM.

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Actin nucleation is one of the key control points in cellular regulation of actin cytoskeleton dynamics. The Arp2/3 complex is an evolutionarily conserved nucleator that binds to the side of an existing actin filament and induces polymerization of a new daughter filament at a 70 degree angle. In many organisms, this 'branched nucleation' activity of Arp2/3 complex is essential for cell motility, endocytosis, and intracellular transport of specific organelles and vesicles. While the biochemical mechanisms leading to activation of the Arp2/3 complex by WASP and WAVE are beginning to be understood in some detail, what is comparatively less clear is how densely branched networks of filaments are subsequently 'pruned' or debranched during phases of rapid actin network turnover and remodeling *in vivo*. Further, there remains a limited understanding of how the many different ligands of Arp2/3 complex affect its conformation and activity. Here, we have used single particle electron microscopy to determine the 3D structures of Arp2/3 complex bound to two recently-identified ligands/regulators, Arpin (1) and GMF (Dang et al., 2013). We found that Arp2/3 complex bound to Arpin adopted an 'open' inactive conformation, and since the binding site on Arp2/3 complex for Arpin

overlaps with the VCA-binding site (Xu et al., 2012), this suggests a specific mechanism of nucleation inhibition. On the other hand, we found that GMF-bound Arp2/3 complex adopted two novel and distinct conformations, possibly reflecting the occupancy of two separate GMF-binding sites on Arp2/3 complex, as proposed (Ydenberg et al., 2013). Additionally, GMF and Coronin together were more effective in shifting Arp2/3 complex to the inactive conformation, and in blocking nucleation activity, demonstrating that multiple ligands can work in concert to govern Arp2/3 conformation and activity.

Literature: Dang, I., Gorelik, R., Sousa-Blin, C., Derivery, E., Guerin, C., Linkner, J., Nemethova, M., Dumortier, J.G., Giger, F.A., Chipysheva, T.A., et al. (2013). Inhibitory signalling to the Arp2/3 complex steers cell migration. *Nature* 503, 281-284. Xu, X.P., Rouiller, I., Slaughter, B.D., Egile, C., Kim, E., Unruh, J.R., Fan, X., Pollard, T.D., Li, R., Hanein, D., et al. (2012). Three-dimensional reconstructions of Arp2/3 complex with bound nucleation promoting factors. *Embo J* 31, 236-247. Ydenberg, C.A., Padrick, S.B., Sweeney, M.O., Gandhi, M., Sokolova, O., and Goode, B.L. (2013). GMF severs actin-Arp2/3 complex branch junctions by a cofilin-like mechanism. *Curr Biol* 23, 1037-1045.

P1624

Board Number: B136

Spectroscopic measurements of conformational changes in Arp2/3 complex.

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Actin filament nucleation and branching by the actin-related protein (Arp) 2/3 complex play key roles in cell migration, endocytosis and cytokinesis. The ubiquitous Arp2/3 complex consists of seven subunits, two of which, Arp2 and Arp3, are structurally similar to actin. The crystal structure of the inactive state of the Arp2/3 complex suggests that a ~30 Å movement of Arp2 towards Arp3 is required for the activation of Arp2/3 complex. Together with actin filaments, the VCA motifs at the C-terminus of nucleation promoting factors (NPFs) are thought to activate and drive Arp2/3 complex conformational changes that are necessary for the branch formation at the side of a pre-existing actin filament. However, how actin filaments and VCA motifs activate Arp2/3 complex is currently unclear, mainly due to the lack of detailed biochemical and structural characterization of conformational changes occurring in Arp2/3 complex activation pathway. Here, we used fluorescence spectroscopy to study interactions of Arp2/3 complex with VCA and actin filaments. We labeled specific sites on Arp2, Arp3, ARPC1 and ARPC3 with fluorescent dyes (FIAsH and Alexa568) either singly or in pairs and studied the effects of VCA and actin filament binding on fluorescence intensity. First, we observed changes in fluorescence intensity in singly labeled Arp2/3 complex. VCA binding to Arp2/3 complex increased the fluorescence intensities of Arp2-FIAsH, Arp3-Alexa568, and ARPC3-FIAsH constructs indicating that this ligand changes the environment of the fluorophores. Intensity changes, directly or through conformational changes, suggest VCA binding sites near the C-terminus of Arp2 and the interface of ARPC3 and Arp3. The

locations on Arp3 and ARPC3 are consistent with previous interpretations of NMR, crystal structures and chemical crosslinking data. The effect of VCA on the fluorescence intensity of Arp2-Flash suggests that C might bind to Arp2 in a similar way, as proposed by Boczkowska et al. and Padrick et al. Second, we observed binding of VCA and/or actin filaments to double-labeled constructs Arp2-Flash/Arp3-Alexa568 and ARPC1-Flash/ARPC3-Alexa568 changed the emission intensity profile of the fluorophores consistent with Förster resonance energy transfer (FRET) efficiency changes induced by each ligand. Our results suggest a model in which VCA and actin filaments contribute distinctly to Arp2/3 complex structural changes

P1625

Board Number: B137

Differential requirement of Arp2/3 complex subunits for actin polymerization during *Listeria* cell invasion and actin comet tail formation ***Listeria* is a bacterial pathogen that subverts mammalian signals.**

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Listeria is a bacterial pathogen that subverts mammalian signaling cascades to infect diverse host tissues. Entry within non-polarized epithelial cells is mainly triggered by interaction between the bacterial protein InlB and the cellular receptor Met, leading to plasma membrane and cytoskeletal rearrangements dependent on the actin nucleator complex Arp2/3. The bacterial protein ActA also activates the Arp2/3 complex for actin-based motility and cell-to-cell spread. Applying a genome-wide siRNA screen for host factors involved in *Listeria* infection, we established that not all subunits of the Arp2/3 complex are equally required for bacterial entry and actin tail formation: while only the subunits Arp2, Arp3, p21, p34 and p41 are required for entry (p16 and p20 are dispensable for this step), all the subunits except p16 are necessary for actin tail formation. Our data highlight that different biological processes may use alternative Arp2/3 complexes.

P1626

Board Number: B138

Interactions with Actin Monomers, Actin Filaments and Arp2/3 Complex Define the Roles of WASP Family Proteins and Cortactin in Coordinately Regulating Branched Actin Networks.

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Arp2/3 complex is an important actin filament nucleator that creates branched actin filament networks required for formation of lamellipodia and endocytic actin structures. Cellular assembly of branched

actin networks frequently requires multiple Arp2/3 complex activators, or nucleation promoting factors (NPFs). We recently presented a mechanism by which cortactin, a weak NPF, can displace a more potent NPF, N-WASP, from nascent branch junctions to synergistically accelerate nucleation rates. The distinct roles of these NPFs in branching nucleation are surprising given their similarities. We biochemically dissected these two classes of NPFs to determine how their Arp2/3 complex and actin interacting segments modulate their influences on branched actin networks. We find that the Arp2/3 complex-interacting N-terminal acidic sequence (NtA) of cortactin has structural features distinct from WASP acidic regions (A) that are required for synergy between the two NPFs. Our mutational analysis shows that differences between NtA and A do not explain the weak intrinsic NPF activity of cortactin, but instead that cortactin is a weak NPF because it cannot recruit actin monomers to Arp2/3 complex. We use TIRF microscopy to show that cortactin bundles branched actin filaments using actin filament binding repeats within a single cortactin molecule, but that N-WASP antagonizes cortactin-mediated bundling. Finally, we demonstrate that multiple WASP family proteins synergistically activate Arp2/3 complex and determine the biochemical requirements in WASP proteins for synergy. Our data indicate that synergy between WASP proteins and cortactin may play a general role in assembling diverse actin-based structures, including lamellipodia, podosomes, and endocytic actin networks.

P1627

Board Number: B139

New insights on the regulation of Las17 during endocytosis.

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During endocytosis, remodeling of the plasma membrane requires a repertoire of endocytic regulatory proteins working in concert to achieve cargo accumulation and vesicle internalization. Clathrin-mediated endocytosis (CME) is a fundamental pathway conserved from yeast to humans that proceeds by forming a clathrin coat at the plasma membrane followed by the recruitment of proteins that promote membrane curvature, actin polymerization, and scission. The Wiskott-aldrich syndrome protein (WASp) and its homologues play a main role in activation of the Arp2/3 complex and actin nucleation in CME. Regulation of WASp family members can be achieved in different ways including phosphorylation-dephosphorylation cycles, autoinhibition, and assembly in to stable complexes where the capacity for promoting actin polymerization is blocked. In budding yeast, Las17 (WASp) is associated in a large and biochemically stable complex with the clathrin adaptor Sla1 (intersectin-1) that we have termed SLAC. Sla1 binds to sequences overlapping a newly identified G-actin binding site in Las17, thus blocking G-actin binding and inhibiting Las17 activity. It has been shown that Bzz1 (syndapin), an F-BAR containing protein, can relieve Sla1 inhibition on Las17 in vitro. Here we present new findings for SLAC complex regulation by Bzz1 and describe a Las17 novel G-actin binding motif (LGM) dependent on a group of arginine residues. We used a combination of biochemical analyses, pyrene-actin polymerization assays, and live cell fluorescence microscopy experiments to demonstrate the importance of a specific interaction between Sla1 and Bzz1 for SLAC activation both in vitro and in vivo. Release of Las17 NPF

activity is essential for normal progression of endocytosis and the data presented here suggest activation occurs through a Bzz1-mediated mechanism, where LGM is accessible to bind actin, without requiring SLAC complex dissociation.

P1628

Board Number: B140

Dissecting contributions of the Arp2/3 complex activators to the endocytic actin patch assembly in fission yeast.

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In yeast and other organisms, endocytosis is dependent upon Arp2/3 complex-mediated actin assembly into endocytic actin patches. Nucleation Promoting Factors (NPFs) control actin assembly by binding and activating Arp2/3 complex via their C-terminal Central-Acidic (CA) domains. In fission yeast, three activators of Arp2/3 complex, Pan1, myosin-1 Myo1, and WASp Wsp1, participate in actin patch assembly. To assess the contributions of these proteins, we tracked the dynamics of actin patches, labeled with GFP-tagged actin binding protein fimbrin Fim1, in pan1, myo1 and wsp1 mutants with deleted CA domains. Deletion of CA domains had no effect on the ability of NPFs to localize to endocytic patches but caused defects in actin patch dynamics specific to each NPF CA deletion. In myo1 CA deletion cells, patch dynamics and internalization was the same as in the wild type cells. In pan1 CA deletion cells, patches internalized normally but accumulated 2.3 times more actin. In wsp1 CA deletion cells, the rate of patch assembly decreased compared to the wild type cells and more than 60% of the patches failed to internalize. To examine patch dynamics in cells that had only one remaining functional NPF, we used genetic crosses to combine two NPF CA deletions in the same cell. While combinations of myo1 CA deletion with pan1 CA deletion or wsp1 CA deletion were viable, the combination of wsp1 and pan1 CA deletions was synthetically lethal. Imaging Fim1-mGFP in cells combining the myo1 CA deletion with pan1 or wsp1 CA deletions revealed the same actin patch dynamics as in cells with single pan1 or wsp1 CA deletions, respectively. Thus, activation of Arp2/3 complex by either Wsp1 or Pan1 alone but not Myo1 alone is sufficient to promote actin patch assembly and cell viability. To directly test the inability of Myo1 to stimulate actin patch assembly, we created a conditionally lethal strain combining pan1 CA deletion with a shut-off or Degron-tagged alleles of wsp1 that allow depletion of Wsp1 from cells. Imaging Fim1-mGFP in these cells revealed a substantial reduction in the number of patches, while few remaining patches had a greatly extended lifetime and failed to internalize. Interestingly, in cells where all three NPFs were disabled by a combination of wsp1 shut-off with both pan1 and myo1 CA deletions, the number of patches and the rate of patch initiation were greatly reduced, although a few patches persisted but failed to internalize. This residual patch assembly may be due to incomplete depletion of Wsp1 or the presence of additional NPFs, such as Dip1. We concluded that Wsp1 is the primary Arp2/3 complex activator at the endocytic sites, minimally sufficient to support actin assembly, endocytic internalization, and cell viability.

P1629**Board Number: B141****In vitro reconstitution of actin filament networks that drive cell motility.**C. Suarez¹, J.D. Winkelman¹, A.J. Harker², D.R. Kovar^{1,2};¹Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL, ²Biochemistry and Molecular Biology, University of Chicago, Chicago, IL

Cells simultaneously assemble and maintain different actin filament networks involved in diverse processes such as cell motility and division. Filopodia are finger-like membrane protrusions proposed to be sensors that determine the direction of cell displacement. Filopodia are composed of long actin filament bundles. It has been hypothesized that filopodia are generated from a dense short, branched and capped network of actin filaments. We propose that the combined action of seven actin binding protein (Arp2/3 complex, WASP-pWA, Ena/VASP, formin, fascin, profilin, capping protein) is necessary and sufficient for their formation. In order to determine if the combination of these proteins is sufficient for the generation of filopodia, we have reconstituted in vitro the self-assembly of filopodia-like networks from these seven purified proteins. To this end, we used innovative techniques such as cover glass surface micropatterning and two-color Total Internal Reflection Fluorescence (TIRF) microscopy. These techniques allow spatial control of actin filament assembly and the continuous observation of fluorescently labeled single proteins. This setup has allowed us to test the role of each component of the system in filopodia formation in a polymerizing network. These experiments revealed that in the presence of a saturating concentration of barbed end capping protein, filopodia-like networks are rapidly facilitated by addition of the processive actin filament barbed end binding competitors formin or Ena/VASP. Our reconstituted system provides a unique platform to follow the assembly of filopodia-like networks upon systematically varying the participating components.

P1630**Board Number: B142****C. elegans apical junction complexes interact with the WAVE/SCAR complex and regulate its stability.**M. . Agapito¹, M. Honarbakhsh¹, Y. Bernadskaya², F. Patel¹, M. Soto¹;¹Pathology, Rutgers-RWJMS, Piscataway, NJ, ²Biomedical Sciences, NYU, Manhattan, NY

During *C. elegans* embryogenesis movements of cells and tissues depend on dynamic reorganization of the actin cytoskeleton. Results from our lab showed that WAVE/SCAR and Arp2/3 are needed for the membrane enrichment of proteins that regulate apical junctions in developing epithelial cells of *C. elegans*. We will present recent results addressing how Arp2/3 and its WAVE/SCAR nucleation-promoting factor help build and maintain apical junctions. Particularly exciting is the finding that WAVE/SCAR components associate with junctional complex components and that loss of WAVE/SCAR components strongly reduces the apical enrichment of alpha-catenin/HMP-1, the protein that connects

the cadherin junction to F-actin. In addition, studies have shown that WAVE/SCAR is phosphorylated at multiple sites, but *in vivo* validation is limited. Using a dephosphorylation assay we determined that the levels of dephosphorylated (and presumably active) WAVE-1 are affected by the junctional proteins. These results provide organismal evidence for the role of branched actin regulators in assembling and maintain the apical junction during embryonic development.

P1631

Board Number: B143

Regulation of Neural Wiskott Aldrich Syndrome (N-WASP) expression under hypoxic conditions.

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Oxygen supply is diminished in highly proliferative, solid tumors especially at the center of the solid tumor resulting in low oxygen tension referred to as hypoxia. Hypoxia inducible factors (HiFs) are heterodimeric transcriptional factors induced under conditions of hypoxia. HiFs consist of two subunits: an alpha subunit and beta subunit. The alpha subunit is regulatory and stable under hypoxic conditions, whereas the beta subunit is expressed constitutively and is also involved in xenobiotic responses. The HiFs regulate the expression of target genes by binding to the hypoxia response element (HRE) present in the promoter of the target genes. Expression of (N-WASP) has been shown to be upregulated under hypoxic conditions. We have found that the activity of N-WASP promoter is enhanced in the presence of hypoxia inducible factor suggesting a possible regulatory mechanism exerted by HiF1 α on N-WASP promoter activity. Sequence analysis of the N-WASP promoter revealed the presence of two HRE elements characterized by the HRE consensus sequence. In order to characterize the regulation of N-WASP promoter by HiF we have generated sited directed mutants of N-WASP promoter in which either of the HRE or both of the HRE have been mutated. The promoter activity of these constructs will be determined to characterize functionality of the two HRE elements. The binding of HiF to the HRE in N-WASP promoter will be determined by EMSA assay to correlate the regulation of N-WASP promoter activity to binding of HiF to HRE in N-WASP promoter.

P1632

Board Number: B144

Effect of aging on thin filament length in mouse skeletal muscle.

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Precise assembly and regulation of the thin and thick filaments (myofilaments) in sarcomeres are vital for determining the biomechanical and physiological properties of skeletal muscle. Since the degree of myofilament overlap determines the amount of contractile force that skeletal muscle sarcomeres can

actively generate, myofilament lengths are accurate predictors of sarcomere operating length ranges. Given the striking uniformity of thin filament lengths specified during myofibril assembly, it is widely assumed that thin filament lengths, and, hence, sarcomere operating length ranges, remain constant throughout the lifespan. However, the effect of aging on thin filament length has yet to be rigorously explored. To examine this problem, we first excised tibialis anterior (TA), extensor digitorum longus (EDL), and soleus muscles from two-month-old and two-year-old mice. Tissue cryosections were then immunostained to label tropomodulin (Tmod) and α -actinin and phalloidin-stained to label F-actin, and imaged using confocal fluorescence microscopy. Background-corrected line scans along myofibrils were then collected from these fluorescence images, and thin filament lengths, defined as Tmod distances from the Z-line, were determined using distributed deconvolution analysis. When comparing young vs. old mice, soleus muscle displayed the greatest difference in thin filament length, followed by the EDL and TA muscles. Young soleus thin filaments were 0.08 μm longer than the old soleus thin filaments ($1.19 \pm 0.07 \mu\text{m}$ vs. $1.11 \pm 0.02 \mu\text{m}$, respectively), young EDL thin filaments were 0.04 μm shorter than old EDL thin filaments ($1.07 \pm 0.07 \mu\text{m}$ vs. $1.11 \pm 0.06 \mu\text{m}$, respectively), and young TA thin filaments were 0.03 μm longer than old TA thin filaments ($1.11 \pm 0.055 \mu\text{m}$ vs. $1.08 \pm 0.07 \mu\text{m}$, respectively). These results identify a novel relationship between aging and thin filament length in skeletal muscle and may provide insight into the mechanisms of altered muscle contractile function in aging. Future investigations will focus on addressing the molecular and cellular basis for thin filament length alterations in aging, such as pathways leading to changes in the relative levels or isoform expression of Tmods, tropomyosins, and other thin filament-associated proteins.

P1633

Board Number: B145

Tks5 localizes to EPEC pedestals and may be involved in initial EPEC attachment to host cells.

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Enteropathogenic *Escherichia coli* (EPEC) is a pathogen that infects the lining of the small intestine causing diarrhea especially in children. EPEC injects several virulence factors into the host cell, including Translocated intimin receptor (Tir), which is inserted into the plasma membrane of the host cell where it serves as a receptor for EPEC. Clustering and phosphorylation of Tir leads to recruitment of actin-regulating proteins that polymerize actin into a pedestal-like structure beneath EPEC. The EPEC pedestal has several similarities with podosomes. Tks5 is an early podosome-specific scavenger protein involved in the formation of new podosomes by interacting with specific phosphoinositides. But despite the similarities of EPEC pedestals and podosomes, Tks5 has so far never been reported to be involved in EPEC pedestal formation. To study Tks5 in the context of EPEC infection, an epithelial MDCK cell line stably expressing Tks5-EGFP was created. Tks5-EGFP was recruited to the EPEC infection site and

localized with actin in the pedestal. The recruitment was Tir dependent, as an EPEC mutant lacking Tir (Δ Tir) was unable to recruit Tks5. In the pedestal, Tks5-EGFP colocalized with actin and N-WASp and time-lapse microscopy revealed that Tks5 was recruited simultaneously with both actin (visualized by LifeAct-mRuby) and N-WASp-mCherry, instantly following EPEC attachment. To elucidate a potential functional role for Tks5 in EPEC attachment, early infection efficiency of EPEC on WT MDCK cells versus MDCK cells overexpressing Tks5-EGFP was examined by a Colony Forming Unit (CFU) assay. After five minutes of infection, a mutant lacking bundle-forming pili had an increased attachment of $70.1 \pm 13.2\%$ to Tks5-EGFP overexpressing cells, compared to WT MDCK cells (p

P1634

Board Number: B146

The Effects of Lipopolysaccharide (LPS) on RING Finger Protein 13 (RNF13) Expression.

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Really Interesting New Gene Finger protein 13 (RNF13) is an E3 ubiquitin ligase that “tags” them for destruction via the proteasome pathway (Rao, Navin et al, 2002). RNF13 mRNA levels are up-regulated upon exposure to the extracellular matrix protein tenascin C (TNC; Midwood, Sacre et al 2009). Since lipopolysaccharide (LPS), like TNC, uses toll-like receptor 4 (TLR-4) signaling pathways (Midwood, Sacre et al 2009), we investigated the hypothesis that LPS upregulates RNF13 expressions in RAW 264.7 macrophages. Using qRT-PCR we demonstrated that RNF13 expression is increased upon LPS stimulation. Fluorescence microscopy was used to examine the localization of RNF13. It co-localizes with Arp2/3p21 in macrophages. The interaction of RNF13 and ARP2/3p21 after LPS stimulation was confirmed by co-immunoprecipitation. Additionally, the data demonstrate that LPS increases the amount of RNF13 isolated with ARP2/3p21. The role of the proteasome was investigated using MG132. Compromising proteasome function in the presence of LPS further increased the amount of RNF13-Arp2/3p21 complex that was detected. The data suggest that the interaction of RNF13 with Arp2/3p21 may facilitate its degradation in a proteasome-dependent manner. The data underscore the potential of RNF13 to impact actin cytoskeleton rearrangement just as its paralog, GRAIL, facilitates the turnover of another member of the complex ARP2/3p16 (Ichikawa, Mizuno, et al 2011).

P1635**Board Number: B147****Ewing sarcoma cell regulation of the cytoskeleton and the impact in a mouse model of tumorigenesis and metastasis.**L.M. Hoffman¹, C.C. Jensen¹, A. Chaturvedi¹, M.C. Beckerle¹;¹Huntsman Cancer Institute, University of Utah, Salt Lake City, UT

Ewing sarcoma is an aggressive pediatric bone cancer that causes osteolytic bone destruction at the primary tumor site and metastatic spread to secondary sites such as the lung. A common chromosomal translocation t(11;22) in Ewing sarcoma generates the oncogenic transcription factor EWS/FLI. Microarray analysis of Ewing sarcoma cells with or without EWS/FLI (using RNAi knockdown) showed EWS/FLI expression repressed many genes, including focal adhesion and extracellular matrix components, and regulators of the actin cytoskeleton. This is consistent with our observation that patient-derived Ewing sarcoma cells in culture were poorly spread cells with ill-defined actin cytoskeletons and that EWS/FLI knockdown led to increased cell adhesion and spreading and robust phalloidin-stained cytoskeletons. Our findings that EWS/FLI down-regulated adhesion-related genes and compromised adhesion and the cytoskeletons in Ewing sarcoma cells led us to consider the hypothesis that these cells are more metastatic because of their poor adhesion in the primary tumor, and suggested that reversal of their cell phenotype could abrogate their metastatic capacity. Two EWS/FLI down-regulated targets are focal adhesion components zyxin and $\alpha 5$ integrin. To investigate their role in Ewing sarcoma cells, retroviral constructs were introduced to reexpress zyxin or $\alpha 5$ integrin, alone or together. Using immunofluorescent microscopy to detect focal adhesions (paxillin localization) and F-actin (phalloidin) we quantitated focal adhesions and cell area. The reexpression of zyxin alone, or with $\alpha 5$ integrin, was sufficient to increase focal adhesions and actin stress fibers, cell spreading and adhesion. To test the consequences of these cell changes in vivo, the engineered Ewing sarcoma cells were tagged with a luciferase reporter and injected into the tibias of immunodeficient mice. In vivo bioluminescent imaging was used to quantitate primary tumor growth in the injected tibias and to detect secondary metastasis. All of the engineered Ewing sarcoma cell types successfully xenografted as primary tumors at the site of injection and showed some ability to metastasize to the lung. Compared to the parent Ewing sarcoma cells, reexpression of zyxin and $\alpha 5$ integrin enhanced cell adhesion and colonization in the lung in both the tibial injection model and with tail vein injections. Oncogene-driven changes may reflect the trade-offs that occur as cells navigate the tumor environment, extravasate, migrate, adhere and re-colonize distant sites. In the future, we envision this in vivo mouse model being used to test therapeutic agents designed to globally reverse the EWS/FLI oncogene-dependent cell phenotype and impair primary tumor growth and/or secondary metastasis.

P1636**Board Number: B148****Differences in Degradation and Distribution of Mutated WWP1 E3 Ubiquitin Protein Ligase between Chicken and Mouse Skeletal Muscles.**M. Imamura¹, S. Takeda¹;¹Department of Molecular Therapy, Natl Inst Neurosci/NCNP, Tokyo, Japan

A missense mutation in a gene encoding WWP1 was identified as the cause of chicken muscular dystrophy by genetic linkage analysis. WWP1 is a HECT-type E3 ubiquitin protein ligase, composed of 922 amino acids, containing four WW domains that interact with proline-rich peptide motifs of target proteins. The mutation changes arginine into glutamine at 441st amino acid located at the center of WW domain cluster, which gives rise to speculation that the amino-acid exchange would effect some change in the function of WWP1. Thus, to test the hypothesis, we generated a mouse model expressing WWP1 transgene carrying the c.1307G>A (p.R436Q) mutation corresponding to the chicken mutation c.1322G>A (p.R441Q) in muscular dystrophy, and characterized the mutated WWP1 in the muscles. In contrast to dystrophic chicken, the transgenic (Tg) mice showed mild muscle phenotype; very few necrotic muscle fibers and adipocytes were observed although significant tubular aggregates were seen with aging. On a molecular level, we also found differences in degradation and distribution of mutated WWP1 in their muscle tissues. WWP1 is normally localized along sarcolemma and sarcoplasmic reticulum (SR) but almost all mutated WWP1s were detected at sarcolemma in mouse muscle, whereas mutated molecules were mainly observed along SR in chicken. Immunoblot analysis clearly showed specific degradation of mutated WWP1s in both animal muscles and that degraded products (90 kDa fragments) of mouse WWP1 were much more stable than those of chicken WWP1. These findings suggested that differences in localization and stability of WWP1-degraded products would relate to different muscle phenotypes between dystrophic chicken and Tg mouse model.

P1637**Board Number: B149****Biodegradable Polymers as Potential Inhibitors for Food-borne Pathogens.**R.W. McClennon¹, C. Carnegie¹, C.D. Johnson¹, E. Williams¹, J.M. Stone¹, S. Woldesenbet¹, L.E. Carson¹;¹Cooperative Agricultural Research Center, Prairie View AM University, Prairie View, TX

Meat packaging has come a long way since the early 1900's post government inspection days instated by the USDA. Due to the advancements in technology and the use of chemicals, we can better protect our food supply, but there is still contamination. This has generated a need to develop a safer natural means of food distribution and packaging without the use of chemicals. Chitosan, a biodegradable polymer found in nature, is one potential packaging material to consider. Chitosan and its derivatives have been shown to have antimicrobial properties. The purpose of this research was to observe the antimicrobial effects of chitosan and its derivatives on the growth of *Listeria*, *Staphylococcus aureus*, and *Salmonella*. These bacteria were selected because they are among the most common food-borne

pathogens. Typically, bacteria was grown in tryptic soy agar (TSA) and incubated at 35°C for *Listeria* and *Staphylococcus aureus* and 37°C for *Salmonella* in separate experiments for 24 hours. Multiple samples of chitosan film were placed on top of the bacteria and agar. Samples were then examined using a zone of inhibition test that is based on the diameter of the clear inhibition zone. The same bacteria samples were then plated, and multiple samples of chitosan powder were placed on top of the bacteria and agar plates. After 24 hours of incubation at 35°C for *Listeria* and *Staphylococcus aureus* and 37°C for *Salmonella*, the zone of inhibition test was conducted. Initial studies indicated that chitosan and its derivatives did not inhibit the growth under conditions in this study. Additionally, chitosan in the powder form had the same results as the film components. While pure chitosan and its degraded derivatives did not inhibit bacterial growth, future studies will focus on using chitosan grafted samples.

P1638

Board Number: B150

The response of bacterial growth rate to osmotic shock.

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Turgor pressure, the hydrostatic pressure within bacterial cells, has long been thought to be an essential factor for their growth by providing the mechanical force necessary for expansion of the peptidoglycan cell-wall. To test this theory, we used microfluidic cell-culture and high-resolution microscopy to precisely measure the elongation rate of bacterial cells while modulating their turgor pressure via osmotic shock. Surprisingly, hyperosmotic shock, which reduces turgor pressure, had no effect on the elongation rate of the cytoplasm of *Escherichia coli* cells, and only reduced the elongation rate of their cell-walls when the osmotic shock was large enough to plasmolyze the cells. Furthermore, when turgor pressure was restored by reversing the shock, the cell wall rapidly elongated to the length that it would have attained had pressure never been depleted. This observation suggested that synthesis of peptidoglycan was unaffected by hyperosmotic shock. In support of this conclusion, we found that the dynamics of MreB, a protein whose motion is correlated with peptidoglycan synthesis, are unaffected by osmotic shock. Therefore, we propose a model of *E. coli* growth in which peptidoglycan synthesis is not driven by turgor pressure, and in which cell-wall expansion is independent of pressure except in the case of plasmolysis. In accordance with this model, hypoosmotic shock, which tends to increase turgor pressure, had little effect on the growth of the Gram-negative *E. coli*. In contrast, hypoosmotic shock had a profound and unexpected effect on the Gram-positive bacteria *Bacillus subtilis*, *Listeria monocytogenes*, and *Clostridium perfringens*. After rapidly swelling during the shock, cells from these organisms displayed damped growth oscillations. We found that this behavior is consistent with a model in which tension within the plasma membrane, induced by the shock, down-regulates peptidoglycan synthesis. Furthermore, our mathematical model correctly predicted the scaling relationships between key variables associated with these oscillations, such as the linear scaling between period of oscillation

and shock magnitude. The model also predicted that reducing membrane tension or peptidoglycan synthesis prior to the shock precludes growth oscillations, which we confirmed experimentally. Currently, we are testing whether there are integral membrane proteins that mediate this process. Finally, in stark contrast to *E. coli*, hyperosmotic shock reduces the elongation rate of *B. subtilis*, suggesting that pressure does drive cell-wall expansion in this organism. That is, whereas the Gram-negative *E. coli* is robust to changes in turgor, Gram-positive bacteria use turgor as part of multiple pathways to control growth.

P1639

Board Number: B151

Synergistic anti-cancer cell impact of anti-tropomyosin compounds with anti-microtubule drugs.

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We have recently developed anti-tropomyosin (ATM) compounds as anti-cancer agents for the treatment of the childhood cancer, neuroblastoma. These compounds target the tropomyosin Tm5NM1 found in most, if not all, human cancers. The ATMs target the N-, C- terminal overlap of adjacent Tm5NM1 dimers in actin filaments. We have demonstrated that the first-in-class (TR100) ATM compound disrupts the actin cytoskeleton of neuroblastoma tumour cells leading to reduced tumour growth in vivo (Cancer Res. 2013 Aug 15; 73(16):5169-5182). Exposure of neuroblastoma cells to ATMs leads to collapse of the actin cytoskeleton and deactivates cell survival pathways. In particular, the p38, MAPK, Akt/PI3K and MEK/ERK pathways are down regulated and there is an increase in the activation of cell death pathways (JNK). Since most cancer therapy involves the use of multiple anti-cancer agents we have evaluated the combinatorial impact of the ATMs with clinically relevant chemotherapeutic agents. Most combinations show additive effects as might be expected of agents which target unrelated signalling pathways and cellular processes. However, the combination of ATMs with anti-microtubule drugs shows striking synergy in the majority of independent neuroblastoma cell lines tested. The extent of synergy varies from 10 to 100 fold. We conclude that targeting the actin and microtubule cytoskeleton may be an effective therapeutic strategy for the treatment of neuroblastoma.

P1640**Board Number: B152****A novel actin filament population defined by the tropomyosin Tm4 regulates ER-to-Golgi trafficking.**E.C. Hardeman¹, L. Yang¹, A.J. Kee¹, E. Polishchuk², R. Polishchuk², I. Pleines³, B. Kile³, R. Weigert⁴, P.W. Gunning¹;¹School of Medical Sciences, UNSW, Sydney, Australia, ²Telethon Institute of Genetics and Medicine, Naples, Italy, ³Water and Eliza Hall Institute, Melbourne VIC, Australia, ⁴NIDCR, NIH, Bethesda, MD

Although the actin cytoskeleton is critical for Golgi morphology and vesicle trafficking, the specific actin filaments associated with ER to Golgi trafficking have not been clearly defined. We have shown that the actin-associated protein tropomyosin (Tm) defines in an isoform-specific manner the function of actin filament populations and that the isoform Tm5NM1 is associated with the Golgi. In this study, we have identified a second Tm isoform, Tm4, at the Golgi that co-localises with β -actin. Tm4 and β -actin localisation is sensitive to the Golgi disrupting drug Brefeldin A (BFA). With BFA removal, Tm4 and β -actin re-association with the Golgi is observed at the early stages of Golgi reformation. In mouse embryonic fibroblasts (MEFs) from a mouse line expressing a mutant Tm4 that is incapable of incorporating into actin filaments, the re-establishment of Golgi morphology after BFA treatment is altered compared with wild-type MEFs. Using the temperature sensitive VSV-G(ts045)-KDEL construct ER-to-Golgi, but not Golgi-to-ER, trafficking was shown to be altered in the Tm4 mutant MEFs. This anterograde trafficking defect is specific to Tm4 as exogenous Tm4, but not Tm5NM1 is able to rescue ER-to-Golgi trafficking in the Tm4 mutant MEFs. Treatment with Blebbistatin, but not CK666 prevented the rescue by exogenous Tm4, demonstrating that Tm4-dependent anterograde trafficking is dependent on Myosin II activity, but not Arp2/3, respectively. Consistent with impaired export from the ER, EM analysis of the Tm4 mutant MEFs revealed a number of ER and Golgi dysmorphologies including the proliferation of swollen ER structures. In conclusion, we have identified a novel Tm4/ β -actin filament population that has an important role in ER-to-Golgi vesicle trafficking.

P1641**Board Number: B153****Tropomyosin Influences on Cellular Functions in Fission Yeast.**B. MacTaggart¹, S. Cranz-Mileva², S.E. Hitchcock-DeGregori²;¹Dept. of Molecular Biology and Biochemistry, Rutgers University, Piscataway, NJ, ²Rutgers Univ-RW Johnson Med Sch, Piscataway, NJ

Tropomyosin (Tpm) binds along actin filaments and regulates actin function. Fission yeast, *Schizosaccharomyces pombe*, contains a single, essential Tpm gene (*cdc8*) that is required for formation of actin cables, the contractile ring and actin patch polarization. *In vitro* Tpm binds actin and regulates myosins, formins and actin filament stability in fission yeast and animal cells. We have tested the function of conserved residues in *cdc8* (Cranz-Mileva et al., 2013; 2013 ASCB abstract). Here we report

on two gene replacement mutants, *cdc8*^{D16A.K30A} and *cdc8*^{E104A}, in which conserved surface residues were mutated to Ala. The mutations have a small or no effect on actin affinity of recombinant Cdc8p, cellular morphology or growth but have distinct effects on the actin cytoskeleton inferring the involvement of the mutated residues in specific cellular functions.

The actin cytoskeleton of *cdc8*^{E104A} (visualized using Lifeact-GFP, LA-GFP in living cells or Alexa-phalloidin in fixed cells) has fewer actin cables and the contractile rings are often wavy or not compact as in wildtype (wt) cells (~29% in *cdc8*^{E104A}, ~2-3% in wt) as observed in strains expressing *rlc1*-mCherry, a myosin II light chain). Patch polarization, visualized using LA-GFP or coronin-GFP is affected; *cdc8*^{E104A} has a greater fraction of cells with an “uneven bipolar” distribution (~45% in *cdc8*^{E104A}, ~25% in wt) with reduced fractions of cells with monopolar patch localization. In addition, the patches are not as compact at the poles. The rate of movement of coronin-GFP patches was reduced by ~30% in *cdc8*^{E104A} cells. We suggest the *cdc8*^{E104A} phenotype reflects delayed restoration of monopolarity after completion of cell division.

Since fission yeast Tpm regulates fission yeast myosin V (Lord et al., 2014; Clayton et al., 2010), we introduced myo52-3xYFP into *cdc8*^{E104A}. As with LA-GFP and coronin-GFP the fraction of monopolar cells was reduced. Since myo52 mediates vacuole distribution (Mulvihill et al., 2001), we visualized the formation and fusion of vacuoles using the dye, FM4-64. In *cdc8*^{E104A} the vacuoles were smaller than wt, but greater in number, inferring deficiency in vacuole fusion.

In another mutant, *cdc8*^{D16A.K30A}, the actin cytoskeleton (Alexa-phalloidin) and fluorescence patterns in strains expressing LA-GFP, *rlc1*-mcherry and Fim-1-GFP were similar to wt. However, the *cdc8*^{D16A.K30A}-Fim-1-GFP cells lack actin cables (visualized using rhodamine phalloidin). Since fimbrin and Tpm compete in binding actin (Skau & Kovar, 2010), we suggest the double *cdc8* mutation and GFP tag on fimbrin have a synthetic negative effect on actin filament assembly.

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P1642

Board Number: B154

New roles for Tropomyosins in controlling formin activities and actin filament dynamics.

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Tropomyosin was one of the first actin-associated proteins discovered (Bailey, 1946, Nature), and plays a prominent role in muscle tissue where it regulates myosin-actin interactions. However, Tropomyosins are also widely expressed in non-muscle tissues, where they have essential roles in a variety of actin-based functions. Based on only a limited amount of biochemical data, dogma has become that

Tropomyosins in non-muscle cells function to stabilize actin filaments and/or block association of other actin-binding proteins. Here, we investigated Tropomyosin function in *S. cerevisiae*, where two isoforms are expressed, Tpm1 and Tpm2, which are genetically redundant and have long been assumed to have similar effects on actin dynamics. Using TIRF microscopy, we uncovered new and differing functional roles for Tpm1 and Tpm2 in actin cable formation. Tpm1 strongly enhanced actin filament nucleation and elongation by the formins Bni1 and Bnr1, both in a profilin-dependent manner. In contrast, Tpm2 inhibited formin-mediated nucleation of actin filaments and had no effect on filament elongation rate. In bulk and TIRF actin disassembly assays, Tpm1 provided some protection of filaments from cofilin-mediated severing, while Tpm2 had no effect. Our results define new roles for Tropomyosins in working with formins to promote actin filament assembly, as well as key mechanistic differences between Tropomyosin isoforms expressed in the same cell type. These biochemical differences between Tpm1 and Tpm2 are supported by genetic differences revealed in directed crosses and through suppression analysis.

P1643

Board Number: B155

Myosin motor isoforms direct specification of actomyosin function by tropomyosins.

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Myosins and tropomyosins represent two types of actin filament-binding proteins that often work together in contractile and motile processes in the cell. While the role of thin filament troponin-tropomyosin complexes in regulating striated muscle myosin-II is well characterized, the role of tropomyosins in non-muscle myosin regulation is not well understood. We previously showed that fission yeast tropomyosin (Cdc8p) positively regulates myosin-II (Myo2p) and myosin-V (Myo52p) motors. To understand the broader implications of this regulation we examined the role of two mammalian tropomyosins (Tm3•1cy/Tm5NM1 and Tm4•2cy/Tm4) recently implicated in cancer cell proliferation and metastasis. Like Cdc8p, the Tm3•1cy and Tm4•2cy isoforms significantly enhance Myo2p and Myo52p motor activity, converting non-processive Myo52p molecules into processive motors that can walk along actin tracks as single molecules. In contrast to the positive regulation of Myo2p and Myo52p, Cdc8p and the mammalian tropomyosins potently inhibited skeletal muscle myosin-II, while having negligible effects on the highly processive mammalian myosin-Va. In support of a conserved role for tropomyosin in regulating non-muscle actomyosin structures, Tm3•1cy rescued normal contractile ring dynamics, cytokinesis, and fission yeast cell growth in the absence of functional Cdc8p. Our work reveals that actomyosin regulation by tropomyosin is ultimately dependent on the myosin isoform. The findings highlight a general role for tropomyosins in gating specific myosin motor outputs. This work has broad implications with regard to sorting of non-muscle and muscle actomyosin function in complex cellular environments such as developing muscle tissue and metastatic cancer cells.

P1644**Board Number: B156****T89 Phosphorylation is a Novel Post-Translational Modification for Critical Structure-Function Regulation of Profilin-1.**D. Gau¹, W. Veon¹, D. Koes², S. Shroff¹, P. Roy¹;¹Bioengineering, University of Pittsburgh, Pittsburgh, PA, ²Computational Biology, University of Pittsburgh, Pittsburgh, PA

Profilin-1 (Pfn1) is a ubiquitously expressed protein which is involved in a wide range of cellular functions including cell migration, proliferation, and control of gene expression. It binds to three classes of ligands including actin, proteins containing poly-L-proline motifs, and membrane phosphoinositides. Dysregulated expression and/or mutation of Pfn1 have been associated with a number of pathological scenarios. Given the importance of Pfn1 in physiology and pathology, we sought to identify novel regulatory mechanisms of Pfn1. We found that Pfn1 exists in multiple post-translationally modified states in cells that are sensitive to phosphatase treatment indicating that these states represent different phosphorylated forms. As a predominant cellular pool of phosphorylated Pfn1 did not involve tyrosine residue, we further investigated potential functional consequences of site-specific serine-threonine (S/T) phosphorylation of Pfn1. We demonstrated that protein kinase A (PKA) can phosphorylate Pfn1 at multiple sites *in vitro* and accordingly, activating PKA pathway modifies Pfn1 *in vivo*. By mass-spectrometry, we identified T89 as a novel phosphorylation site of Pfn1. Phosphomimetic mutation on T89 makes the mutant Pfn1 greatly insoluble, dramatically lowers the protein stability of Pfn1, and affects the actin cytoskeleton. Finally, molecular dynamics simulation of wild-type vs. phosphomimetic variant of Pfn1 predicted that T89 phosphorylation causes a major conformational change in an important loop structure of Pfn1 with potential consequence on its actin-binding. In summary, our studies suggest that T89 could be a novel phosphorylation site for critical structure and function regulation of Pfn1.

P1645**Board Number: B157****Kinetics of profilin-1 and periplakin in a human tongue cancer cell line.**I. Tamura¹, A. Kamada¹, S. Goda¹, Y. Yosikawa¹, E. Domae¹, T. Ikeo¹;¹Osaka Dental Univ., Hirakata-shi, Japan

Profilin-1 is an actin-binding cytoskeletal protein that acts in the regulation of actin filament dynamics. Periplakin is a 195-kDa protein belonging to the plakin family of cytoskeletal linker proteins and is generally localized to the cell membrane of normal stratified squamous epithelia. There is little information on the kinetics of profilin-1 and periplakin in oral primary cancer cells. We investigated the expression of profilin-1, periplakin and cytokeratin 18 in a human tongue cancer cell line (BICR31) and examined the relationship between their expression and oral cancer cell malignancy. The human

tongue keratinocyte line DOK and BICR31 were cultivated to sub-confluence in Dulbecco's Modified Eagle's Medium including 10% fetal bovine serum and 5 µg/ml hydrocortisone, and expression levels of profilin-1, periplakin and cytokeratin 18 were determined by Western blotting analyses and FITC- or RITC-labeled immunocytochemical staining. Moreover, BICR31 cells were split using a pipette chip on the chamber slide, incubated for one, two or three days, then immunocytochemically stained for profilin-1, periplakin and cytokeratin 18. Although an obvious band for profilin-1 was detected by Western blotting analyses in DOK cells, immunoreactions for profilin-1 were weak in BICR31 cells. In addition, anti-profilin-1 antibody reacted with DOK cells, and weakly and diffusely with cytoplasm in BICR31 cells. Conversely, expression of periplakin in most DOK cells was diffuse, and strong reactions were detected in BICR31 cells. For periplakin, there were correlations between Western blotting data and immunocytochemical observations. The kinetics of cytokeratin 18 were similar to those of periplakin. Although there were no changes in the expression of profilin-1 and cytokeratin 18, expression of periplakin tended to increase near the cell layer in the wound model. These results indicate that decreased plectin-1 expression is an index of malignancy, that periplakin is expressed by tongue epithelia during various stages in malignancy associated with the proliferation and migration of cancer cells, and that expression of cytokeratin 18 may show an epithelial-to-mesenchymal transition in squamous epithelial cells.

P1646

Board Number: B158

Drebrin modulates the effects of formins on actin dynamics.

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Drebrin A, a neuronal F-actin-binding protein enriched in dendritic spines, plays a major role in synaptic plasticity. Molecular cross-talk between drebrin and key neuronal actin-binding proteins is believed to be a part of the cytoskeleton-modulating pathway that is implicated also in synaptic dysfunction in Alzheimer's disease (AD), Down syndrome, epilepsy and normal aging. We documented that drebrin acts as a leaky capper of actin filaments. In this context, we investigated here drebrin interrelation with formins – the B-end actin binding factors. We found that drebrin inhibited mDia- and mDia2-assisted polymerization of actin. TIRF microscopy revealed that drebrin introduced heterogeneity into actin filaments population nucleated by mDia1 and mDia2 formins by reducing the fraction of fast-elongating (formin bound) filaments. This effect was not observed with INF2 formin which has also the unique feature of severing actin filaments. We documented here that drebrin strongly inhibited F-actin severing by INF2 formin and investigated the mechanism of such inhibition employing electron microscopy. We found that drebrin allowed for the side binding of INF2 to actin filaments but changed it to an isolated,

single-dimer binding mode, in contrast to continuous decoration of filaments by INF2 alone. The mechanism of formins inhibition by drebrin and its biological implications will be discussed.

P1647

Board Number: B159

Formin FH2 domains accelerate ATP hydrolysis prior to polymerization: insights into formin-specific effects on actin structure.

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Formins are a class of actin assembly factors, and the 15 mammalian formins have wide-spread roles in many actin-based processes. Central to their effects on actin is the dimeric FH2 domain, which can accelerate nucleation of new actin filaments, then modulate elongation by remaining bound to the filament barbed end. There is considerable variation between FH2 domains in both nucleation and elongation efficiencies, and one formin, INF2, can actually enhance depolymerization. While near-atomic resolution structures of actin-bound FH2 domains are available, we do not understand how FH2 domains change actin to enhance nucleation, elongation and, in the case of INF2, depolymerization. Here, we show that formin FH2 domains cause an acceleration of actin's ATPase activity even when actin is in the monomeric state (bound to latrunculin). The magnitude of this acceleration scales roughly with the nucleation efficiency of the FH2 domain, with FH2 domains that potently nucleate being the most acceleratory to ATP hydrolysis from actin monomers. This effect had been predicted for the barbed end-capping molecule, cytochalasin D (Goddette & Frieden (1986) J. Biol. Chem.), and we recapitulate these results to show that FH2 domains act in a quantitatively similar manner. For some formins, the region C-terminal to the FH2 provides further acceleration of ATP hydrolysis. We use analytical ultracentrifugation and electron microscopy to show that actin is not polymerized under these conditions. In the case of INF2, increased ATP hydrolysis rate appears to play a role in its depolymerization activity. When added to pre-polymerized filaments, INF2 causes rapid depolymerization to a state in which filaments are no longer present, yet the system continues to hydrolyze ATP in the non-filamentous state. In summary, we reveal a fundamental effect of FH2 domains, to enhance the ATPase activity of actin monomers, suggesting that formins convert the actin monomer to the "flattened" ATPase-competent conformation (Mizuno et al (2010) Science) prior to its addition to the barbed end. Individual formins such as INF2 can tailor this activity for their specific purposes.

P1648**Board Number: B160****Identification of formin-regulating sequences common to Smy1 and other cytoskeletal proteins.**J.A. Eskin¹, A. Rankova¹, S.L. Alioto¹, A. Johnston¹, B.L. Goode¹;¹Department of Biology, Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, MA

In budding yeast, formins play an essential role in assembling actin cables, which serve as polarized tracks for myosin-based transport of secretory vesicles and other cargoes to the developing bud cortex. We previously showed that the myosin passenger protein Smy1, a motor-inactive Kinesin-1 homologue, directly binds and inhibits the formin Bnr1 to control actin cable length and function (Cataldo et al., 2011). However, Smy1 is a multi-functional protein and also binds F-actin and promotes myosin V (Myo2) processivity in vitro, and thus it has remained unclear whether the abnormal actin cables and defects in secretory traffic in *smy1Δ* cells result from misregulated Bnr1 activity. Here, we have identified three specific sequences in Smy1 that are required for its inhibitory effects on Bnr1 in vitro. In cells, replacement of the wild type *SMY1* gene with *smy1* alleles mutated at these sites led to defects in actin cable formation and secretory vesicle traffic similar to *smy1Δ*. Further, the mutant *smy1* alleles were pseudo-wild type for suppression of *myo2-66* growth defects. Thus, our new *smy1* alleles uncouple Smy1 functions, and demonstrate that Smy1 indeed controls Bnr1 activity in vivo. In addition, BLAST searching with the formin-regulating sequences in Smy1 identified related motifs in yeast Bud14, which is also a Bnr1 regulator (Chesarone et al., 2009). Mutation of the Smy1-like motif in Bud14 impaired its regulatory effects on Bnr1 in vitro, and compromised *BUD14* function in vivo. Further, Smy1-like motifs were identified in several mammalian proteins implicated in formin regulation.

P1649**Board Number: B161****A novel actin-adhesion structure involved in nuclear positioning requires the formin FMN2.**C.T. Skau¹, C. Waterman¹;¹National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD

Active asymmetric positioning of the nucleus in interphase cells is critical to cell functions including cell migration, particularly in complex 3D environments. Previous studies have highlighted the importance of integrin and Rho signaling in controlling nuclear orientation in migrating fibroblasts. Although these studies establish that integrins, actin, and actin-binding proteins at the nuclear envelope are critical for nuclear positioning in adherent cells, it is not known if this is mediated by linking the nucleus to the previously-characterized adhesion/actin system used for migration, or if nucleus-ECM coupling is accomplished via a specialized cytoskeletal system. We tested the hypothesis that a dedicated adhesion-

actin system is responsible for maintenance of nuclear position in adherent, migrating cells. We examined the interplay between actin, adhesions, and the nucleus in fibroblasts using fluorescence microscopy. We identified novel adhesive structures located underneath the nucleus termed subnuclear adhesions that are compositionally and dynamically distinct from the canonical focal adhesions at the leading edge. First, subnuclear adhesions have reduced levels of signaling and actin-binding proteins found in leading edge adhesions but have high levels of fibrillar adhesion proteins, although they are not required for fibrillogenesis. Additionally, unlike focal adhesions at the protruding edge, assembly of the long-lived subnuclear adhesions is coordinated with movement of the nucleus and independent of the leading edge. Furthermore, evidence from nuclear displacement experiments indicates that the number and placement of these adhesions is controlled by the nucleus. We also show that a specific set of actin fibers connecting two subnuclear adhesions can control nuclear shape by physically impinging on the nucleus. These subnuclear fibers have elevated levels of the IIB isoform of myosin as compared with dorsal stress fibers, and are less dependent on the contractile activity of myosin than dorsal stress fibers. We previously identified the actin nucleation factor formin FMN2 in a screen of adhesion components. We now find that FMN2 localizes underneath the nucleus, and that the rapid dynamics of FMN2 partially depend on myosin activity. Critically, FMN2 is essential for both subnuclear actin and adhesions. Cells lacking FMN2 and subnuclear adhesions migrate poorly in 3D environments and exhibit defects in nuclear positioning, including failure to reorient during wound healing. Together, our data reveal the critical role of an actin nucleator in a previously unidentified mechanism for control of nuclear position via a novel adhesive structure linking the actin cytoskeleton, which connects to the nucleus, to the extracellular matrix.

P1650

Board Number: B162

Nuclear actin counters gravity during cell growth.

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Mechanics is highly influential in determining cell behavior from cell division to cell motility. However, the exact role of mechanics in controlling cell growth and cell size still remains unclear. The oocytes or immature eggs of the frog *X. laevis* are known to grow and reach extraordinarily large sizes of approximately 1 mm in diameter. During this process of growth, the oocytes actively maintain significantly high concentrations of nuclear actin. We have recently shown that this nuclear actin meshwork stabilizes the liquid-like nuclear bodies from rapid gravitational sedimentation and fusion. However, the mechanical properties of nuclear actin remain poorly characterized, despite their apparent importance in kinetically stabilizing the nucleus. Here, we use approaches in active and passive microrheology, in addition to confocal and two-photon excitation microscopy and quantitative image analysis, to probe the local mechanics and microstructure of this nuclear actin network. We find that actin forms a surprisingly soft, viscoelastic scaffold with a mesh size of ~0.5 microns. Furthermore, the

distribution of nuclear bodies within the actin network shows signatures of gravitational creep during growth. Upon application of forces of ~ 1 pN, nuclear actin exhibits shear-thickening behavior, which could serve a protective role in response to shocks. However, significantly higher forces ultimately lead to mechanical failure and rupture of the actin network. These measurements elucidate mechanical and geometric aspects of cell organization, which suggests that biophysical constraints can play an important role in cell growth and size control.

P1651

Board Number: B163

Mechanism of actin filament nucleation by Leiomodin.

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Actin cytoskeleton remodeling requires proteins called nucleators, which catalyze the kinetically unfavorable step of new filament formation. Among known filament nucleators Leiomodin (Lmod) is the only one specifically expressed in muscle cells. Lmod, a strong nucleator, is related to the F-actin pointed-end capping protein tropomodulin (Tmod), with negligible nucleation activity. Both proteins contain a flexible N-terminal region and a leucine-rich repeat (LRR) domain. It has been proposed that the difference in their functions originates in the presence in Lmod of a unique C-terminal extension harboring a WH2 domain, an actin barbed end-binding site. Here, we present data in support of a hypothesis that the difference between nucleation and capping activity is defined within the LRR domain. Thus, we designed a hybrid Tmod-Lmod LRR domain with nucleation activity similar to full length Lmod. On the other hand, the addition of Lmod's C-terminal extension to Tmod did not create a strong nucleator.

P1652

Board Number: B164

Structural organization of contractile ring constituents in cortices isolated from dividing sea urchin embryos.

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The sea urchin zygote has long served as a model system for studying changes in the cortical actin cytoskeleton, from the post-fertilization development of microvilli to the organization of the contractile ring. However, in the several decades since much of the seminal work was performed on egg cortices, great strides have been made in our understanding of how actin polymerization is facilitated, how actin filaments are organized, and how the contractile ring is assembled and regulated. In light of these

advances, we have begun to revisit the cortical actin cytoskeleton in isolated cortices using sophisticated light and electron microscopy incorporating probes specific for crucial constituents including actin, total and activated myosin II, the Arp2/3 complex, formin, and RhoA. Examination of the two principle actin nucleating factors (formin2 and the Arp2/3 complex) revealed that in unfertilized cortices, Arp2/3 appeared to associate with small collections of actin filaments inside the short microvilli. In contrast, formin2 was associated with elongate microvilli following fertilization. Examination of the contractile ring in isolated cortices indicated that while myosin II was found throughout the cortex, activated myosin (phospho-serine 19) formed a discrete band at the cell equator. 3D SIM super-resolution microscopy indicated that the activated myosin and actin filaments in the contractile ring aligned in parallel arrays. We have begun to investigate the ultrastructural organization of the contractile ring using critical point dry and rotary shadow TEM of isolated cortices. Imaging of unextracted and detergent extracted cortices showed a distribution of submembranous, elongate actin filaments. In areas outside of the cleavage furrow these filaments were organized into an isotropic network containing nodes whereas within the cleavage furrow they formed a very dense, anisotropic array of elongate filaments. To our knowledge these images offer some of the first 3D ultrastructural views of the contractile ring in animal cells and we are in the process of additional experimentation to further characterize this preparation.

P1653

Board Number: B165

Role of BMP signaling on cytoskeleton elements in the sensory placode invagination.

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Sensory placodes (lens, olfactory, and otic) give rise to sense organs of the vertebrate head. Despite their diversity in shape and function, the sensory organs share common morphological and molecular features during early steps of morphogenesis. The first sign of ectodermal organ formation is a local thickening of the epithelium, the so called placodes, which is accompanied by condensation of the underlying mesenchyme (placode invagination or bending). It is known that cells in placode elongate along their apical-basal axis, simultaneously change cell shape from cylindrical to conical shape due to reduction of apical cell surface (also called apical constriction). Apical constriction is at least partly due to the apically localized acto-myosin cytoskeleton. Molecular nature of the extracellular signals, and when and how they interact to convert flat ectoderm to invaginated placodes remain poorly understood. The communication between and within the two tissues, mediated by several families of signaling molecules including FGFs, Wnts, BMPs, and Notch are expressed in or around the developing sensory placodes. In our study we show that BMP signals appears to be important for the correct positioning of the olfactory, otic and lens placode, as well as for proper invagination, in part by positively regulating the expression of Six1 in the olfactory placode. BMP is also shown to play an important role on cytoskeleton elements in sensory placode invagination.

P1654**Board Number: B166****CAP2 in cardiac conduction, sudden cardiac death and eye development.**J. Field¹, D. Ye¹, M. Shinde¹, F. Liu¹, K. Schillinger¹, M. Lu¹, T. Wang¹, M. Skettini¹, A. Brice¹, D. Chung¹, V. Patel¹;¹University of Pennsylvania, Philadelphia, PA

Sudden cardiac death kills 180,000 to 450,000 Americans annually, predominantly males. A locus that causes developmental disorders and confers a risk for sudden cardiac death and conduction disease is located at 6p22, near the cytoskeletal gene, CAP2. CAP2 regulates actin dynamics in the cytoskeleton by binding actin monomers and assisting filament severing to modulate the balance of filamentous actin (F-actin) and globular actin (G-actin). To determine the role of CAP2 in vivo, we generated knockout (KO) mice. *cap2*⁻/*cap2*⁻ males were underrepresented (Mendelian ratios 5%, expected 25%) and ~70% died by 12 weeks of age, but *cap2*⁻/*cap2*⁻ females were found at close to the expected levels (17%) and lived normal lifespans. CAP2 knockouts were smaller, developed microphthalmia, cardiac conduction disease and dilated cardiomyopathy past 12-weeks of age, most noticeably in the males. To address the mechanisms underlying these phenotypes, we used Cre-mediated recombination to knock out CAP2 in cardiomyocytes and found that the mice died suddenly due to complete heart block in the absence of cardiomyopathy but no longer displayed the other phenotypes, including any evidence of sex-bias. These studies establish a direct role for actin dynamics in sudden cardiac death and cardiac conduction disease.

P1655**Board Number: B167****Drosophila Spire functions as a nucleation promoting factor (NPF) for formin-mediated actin assembly.**C.L. Vizcarra¹, R. Jaiswal², B.L. Goode², M.E. Quinlan^{1,3};¹Chemistry and Biochemistry, University California-Los Angeles, Los Angeles, CA, ²Department of Biology, Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, MA,³Molecular Biology Institute, University California-Los Angeles, Los Angeles, CA

Actin nucleators must be tightly regulated in vivo, which is achieved through mechanisms involving both inter- and intra-molecular interactions. A number of actin nucleators also bind to nucleation promoting factors (NPFs) to fully realize their actin nucleation activity. The best characterized of these nucleator/NPF pairs is Arp2/3 complex/N-WASP, which catalyzes the formation of branched actin filament networks. Recently, it was shown that formins can also collaborate with NPF-like proteins, including the yeast formin Bni1 and Bud6 (Graziano et al., 2011) and the mammalian formin mDia1 and Adenomatous polyposis coli protein (Okada 2010). In vivo, the Drosophila formin Cappuccino (Capu) is associated with a cytoplasmic actin mesh that is essential for oocyte development. Mutation of Capu

leads to loss of the mesh, mislocalization of polarity cues, and female sterility. Mutation of the WH2-based actin nucleator Spire has a similar phenotype in the fly oocyte. In addition, direct interactions between Spire and Capu are essential for actin mesh formation and, subsequently, for oogenesis and fertility (Quinlan 2013). Spire contains four WH2 domains (A, B, C, and D) that each bind actin monomers. In the absence of formins, Spire mutated at WH2-D (Spire-D*) fails to nucleate actin assembly in vitro, similar to a construct with all four WH2 domains mutated (Spire-A*B*C*D*). However, in vivo Spire-D* is able to rescue fertility, while Spire-A*B*C*D* is not, suggesting that Spire's actin nucleation activity on its own may not be necessary in the oocyte. Combined with earlier studies, we conclude that Spire in vivo function during oogenesis requires at least some functional actin monomer binding sites and the ability to interact with Capu. This points to a role for Spire as an NPF for Capu. Indeed, in bulk in vitro assays, Spire stimulates Capu's actin assembly activity in the presence of capping protein and profilin, conditions under which Spire alone does not promote filament formation. To better understand the workings of these two proteins, we are defining the mechanisms underlying actin filament formation by Spire and Capu, alone and together, using multi-wavelength single molecule TIRF.

P1656

Board Number: B168

The role of Rtt102- and ATP-binding to Arp7/9 in regulating SWI/SNF remodeler structure and function.

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Chromatin-remodeling complexes (remodelers) modulate chromatin structure and thus play important roles in regulating transcription, DNA replication, and DNA repair. Remodelers are assembled around a catalytic subunit that contains a central ATPase domain and flanking sequences that recruit auxiliary subunits. The catalytic subunits of SWI/SNF remodelers recruit actin-related proteins 7 and 9 (Arp7/9) through a helicase-SANT-associated (HSA) domain N-terminal to the ATPase domain. We previously showed that an additional auxiliary subunit, Rtt102, binds with nanomolar affinity to the Arp7/9 heterodimer. This induces a conformational change in Arp7/9, which then interacts with a shorter segment of the HSA domain. In addition, Rtt102 promotes high-affinity ATP binding to one of the Arps in the heterodimer. Here, we used x-ray crystallography to further investigate the role of Rtt102-binding on the structure and function of Arp7/9. These studies reveal the binding site for ATP within Arp7/9 while also confirming our previous findings regarding the Rtt102-induced conformational change in Arp7/9. We also present DNA-binding experiments that suggest a role for Rtt102- and ATP-binding to Arp7/9 in regulating SWI/SNF remodeler function.

Higher-Order Actin-Based Structures

P1657

Board Number: B169

Identification of Novel Components of the Intermicrovillar Adhesion Complex that Regulates Intestinal Brush Border Assembly.

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Intestinal enterocytes remodel their apical surface during differentiation to form a brush border: a tightly packed array of microvillar membrane protrusions that exhibit strikingly uniform length and highly organized packing. Although the brush border is essential for maintaining physiological homeostasis in the gut, how enterocytes generate, stabilize, and organize microvilli remains an open question. Our lab recently discovered an adhesion-based mechanism that regulates brush border formation. During brush border assembly, microvilli cluster together on the apical surface of enterocytes through interactions between their distal tips. Adhesion is mediated by two protocadherin family members, protocadherin-24 (PCDH24) and mucin-like protocadherin (MLPCDH), which are found specifically at the distal tips of microvilli. By physically linking neighboring microvilli together, intermicrovillar adhesion promotes the tight packing of microvilli during enterocyte differentiation and enforces uniform length across the brush border. We previously used a candidate approach to identify cytoplasmic molecules that interact with PCDH24 and MLPCDH, including the scaffolding protein harmonin-a and the actin-based motor, Myo7b. Together, these molecules form the intermicrovillar adhesion complex (IMAC). Towards the goal of identifying additional members of the IMAC, we developed a novel protein isolation strategy that takes advantage of the inherent adhesion activity of microvillar protocadherins, to purify intact IMAC from brush borders isolated from native intestinal tissue and polarized CACO-2_{BBE} monolayer cultures. Here we present data on the characterization of novel IMAC components including: (1) their subcellular localization in native intestinal tissue and CACO-2_{BBE} cells (2) their functional role in promoting microvillar clustering and brush border formation using knockdown and overexpression studies performed in CACO-2_{BBE} cells, and (3) their interactions with other IMAC components using pull-down analyses with recombinant proteins. In summary, our data suggest the IMAC is a large multi-protein complex that resides at the tips of brush border microvilli, where it plays a central role in promoting normal brush border formation.

P1658**Board Number: B170****The actin nucleator Cordon-Bleu plays a role in building enterocyte brush border microvilli.**N.E. Grega Larson¹, M.J. Tyska¹;¹Cell and Developmental Biology, Vanderbilt University Medical Center, Nashville, TN

The absorptive epithelial cells that line the small intestine, also known as 'enterocytes', exhibit remarkable apical-basal polarity. The apical surface of the enterocyte displays an array of tightly packed microvilli referred to as the brush border. A single microvillus is a membrane protrusion supported by a linear F-actin bundle, with the plus ends of actin filaments oriented toward the distal tip. Despite the importance of the brush border in nutrient absorption and host defense, the mechanism of brush border assembly has remained unclear. A previous proteomic analysis of isolated brush borders performed by our laboratory identified two actin nucleators in brush border-enriched fractions: the Arp2/3 complex and Cordon-Bleu (COBL). Inhibition of the Arp2/3 complex had no impact on brush border assembly in Ls174T-W4 (W4) cells, which act as a single cell model of enterocyte polarization and differentiation. However, we found that the linear actin nucleator, COBL, localizes to the base of the brush border in rat and mouse small intestine, and in W4 cells. COBL is thought to nucleate F-actin polymerization using three C-terminal WH2 domains that each bind G-actin. shRNA knockdown of COBL in W4 cells causes a significant decrease in the fraction of cells that form brush borders. This effect was fully rescued with a COBL variant that was refractory to knockdown, and partially rescued by a C-terminal fragment containing the G-actin binding WH2 domains. Overexpression of full-length and truncated forms COBL accentuated brush border morphology, with microvilli that were longer, straighter, and contained more F-actin. Moreover, a COBL construct that is unable to bind actin did not rescue COBL knockdown or impact brush border morphology when it was overexpressed, suggesting that actin binding is critical for COBL's role in brush border assembly. To examine how COBL impacts actin dynamics in live cells, we introduced GFP-tagged COBL into B16F1 melanoma cells. Overexpression of COBL in these cells induces the formation of linear actin bundles throughout the cytoplasm. Strikingly, these bundles are highly dynamic, growing and shrinking over the course of two to three minutes. Moreover, COBL is enriched at slow/non-growing end of the bundle (presumably, the minus ends). Finally, these bundles can be stabilized to effect protrusion at the cell periphery by co-expressing F-actin bundling proteins that are normally found in the microvillus, such as espin. Together, these data suggest that COBL plays a role in building brush border microvilli, most likely by nucleating actin filament polymerization from the terminal web.

P1659**Board Number: B171****Endogenous species of mammalian nonmuscle myosin IIA and IIB include activated monomers and heteropolymers.**M. Shutova¹, W.A. Spessott², C.G. Giraudo³, T. Svitkina¹;¹Department of Biology, University of Pennsylvania, Philadelphia, PA, ²Children's Hospital of Philadelphia, Philadelphia, PA, ³The Children's Hospital of Philadelphia - University of Pennsylvania, Philadelphia, PA

Class II myosins generate contractile forces in cells by polymerizing into bipolar filaments and pulling on anchored actin filaments. Nonmuscle myosin II (NMII) plays central roles during cell adhesion, migration, cytokinesis, and tissue morphogenesis. NMII is present in virtually all mammalian cell types as tissue-specific combinations of NMIIA, NMIIB, and NMIIC isoforms. It remains poorly understood how the highly dynamic NMII-actin contractile system begins to assemble at new cellular locations during cell migration and how incorporation of different NMII isoforms into this system is coordinated. Using platinum replica electron microscopy in combination with immunogold labeling, we demonstrate that individual activated (phosphorylated on the regulatory light chain and unfolded) NMIIA and NMIIB molecules represent a functional form of NMII in motile cells and that NMIIA and NMIIB copolymerize into nascent bipolar filaments during contractile system assembly. Using subdiffraction STED microscopy together with a pharmacological block-and-release approach, we report that NMIIA and NMIIB simultaneously incorporate into the cytoskeleton during initiation of contractile system assembly, whereas the characteristic rearward shift of NMIIB relative to NMIIA is established later in the course of NMII turnover. We show existence of activated NMII monomers in cells, copolymerization of endogenous NMIIA and NMIIB molecules, and contribution of both isoforms, rather than only NMIIA, to early stages of the contractile system assembly. These data change the current paradigms about dynamics and functions of NMII and provide new conceptual insights into organization and dynamics of the ubiquitous cellular machinery for contraction that acts in multiple cellular contexts.

P1660**Board Number: B172****Investigation of beta-Spectrin function in the Hippo signaling pathway.**Y. Yan¹, K. Wong¹;¹Division of Life Science, HKUST, Hong Kong, Hong Kong

The Hippo signaling pathway is an evolutionarily conserved pathway regulating a wide range of developmental and cellular processes such as growth, differentiation, and morphogenesis. Importantly, the Hippo signaling pathway is known to respond to regulatory signals from the actin cytoskeleton. However, the mechanism of how the actin cytoskeleton regulates the Hippo pathway remains largely elusive.

We identified beta-Spectrin as a protein required for Hippo signaling activity in a genetic screen for oocyte polarity defects during *Drosophila* oogenesis. Spectrins are known for linking the actin cytoskeleton to the plasma membrane. In beta-spectrin mutant cells the actin filaments lose the planar polarized orientation and frequently form abnormal stress-fiber-like structures on the basal side of the epithelium. Increased cellular tension associated with formation of stress fibers is likely a critical link between the beta-spectrin mutations and the Hippo signaling defect. Moreover, the function of beta-Spectrin in the Hippo signaling pathway is likely to be conserved in the mammalian cells.

P1661

Board Number: B173

Effects of a putative male contraceptive on the ultrastructure of actin filament bundles in the seminiferous and other epithelia.

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H2-Gamendazole is a non-hormonal compound being actively investigated as a male contraceptive. It targets eEF1A, a protein synthesis factor that also influences the arrangement of actin filaments. In the testis, the drug causes loss of intercellular adhesion and detachment of developing sperm cells. Ectoplasmic specializations (ESs) are Sertoli cell specific adhesion junctions that consist of a layer of actin filaments situated between the plasma membrane and a cistern of endoplasmic reticulum. The filaments are unipolar in orientation and packed into hexagonal arrays, like the arrangement of filaments in microvilli. Here, we explore the effects of H2-gamendazole on ESs, and determine if drug-related effects occur in the microvilli of other epithelial cell types. Rats were given a single oral dose of H2-gamendazole (30 mg/Kg). This dose is near the maximum tolerated dose and is 10-20x the expected dose for contraceptive use. It was chosen to maximize any potential effects in off-target tissues. At 6 & 24 hrs after dosing, samples of testis, small intestine, large intestine, and kidney from control and treated animals were processed for electron microscopy. Actin filaments in ESs were disrupted in drug treated animals compared to controls. In addition, microvilli in other tissues were short, developed an irregular shape, and in some cases completely lost their actin cores. Our data are consistent with the conclusion that H2-gamendazole causes premature detachment of spermatogenic cells by targeting the actin cytoskeleton in ESs. Our results also indicate that the drugs effects at a high dose are not absolutely restricted to the testis.

P1662**Board Number: B174****F-actin and Golgi rearrangement around the *Chlamydia trachomatis* inclusion is mediated by the Arf-recruiting bacterial protein InaC.**M. Kokes¹, R.H. Valdivia²;¹Molecular Genetics and Microbiology, Cell and Molecular Biology, Duke University, Durham, NC,²Molecular Genetics and Microbiology, Duke University Medical Center, Durham, NC

Chlamydia trachomatis is the most common sexually transmitted bacterial pathogen, and is the leading cause of preventable blindness worldwide. As an obligate intracellular pathogen, *Chlamydia* resides within a membrane-bound vacuole (inclusion) within host cells. A filamentous actin structure surrounds the inclusion, and disruption of actin polymerization in host cells leads to increased exposure to cytosolic innate immune receptors and increased expression of pro-inflammatory cytokines, suggesting that F-actin maintains *Chlamydia* in a 'hidden' state within cells.

To identify *Chlamydia* factors involved in cytoskeletal cage assembly at inclusions, we screened an arrayed library of chemically-mutagenized strains and identified one mutant lacking F-actin at inclusions. Using genetic tools recently developed for *Chlamydia*, we show that a secreted bacterial protein associated with the cytosolic surface of the inclusion membrane, renamed InaC, is necessary for actin assembly at the inclusion. Surprisingly, InaC is dispensable for suppression of host cytokine production. To further define roles of InaC in modifying the host intracellular environment, we used a combination of immunoprecipitation and mass spectroscopy to identify host proteins that interact with InaC. We show that InaC interacts with and recruits host Arf GTPases – important regulators of trafficking and organization at the Golgi – to the inclusion. Remarkably, during *Chlamydia* infection, the Golgi ribbon fragments into ministacks which rearrange around the periphery of the inclusion. We find Golgi re-distribution around the inclusion to be mediated by InaC in an F-actin-dependent manner. Sphingolipids normally trafficked from the Golgi to the plasma membrane are intercepted by *Chlamydia*, and host sphingolipids are incorporated into bacteria and the inclusion membrane. Golgi re-distribution around the inclusion has previously been proposed to enhance bacterial acquisition of host sphingolipids. However, we find that InaC-deficient *Chlamydia* lacking actin assembly at the inclusion acquire normal levels of sphingolipids, suggesting an alternative role for InaC-mediated Golgi re-distribution around the inclusion.

P1663**Board Number: B175****Acetylation of K326 and K328 of actin depresses contractile performance of skeletal and cardiac muscle.**M.C. Viswanathan¹, A. Blice-Baum¹, D.B. Foster¹, A. Cammarato¹;¹Medicine, Johns Hopkins University, Baltimore, MD

Striated muscle contraction results from transient interactions between myosin-containing thick and F-actin-containing thin filaments. Tropomyosin (Tm) extends along the F-actin thin filament surface. Its location governs access of myosin binding sites and, hence, force production. Models of the F-actin-Tm and F-actin-Tm-myosin binding interfaces have been proposed based on evolutionary analysis, computational chemistry, and image reconstructions. Intermolecular electrostatic associations mediate critical interactions between the proteins. For example, actin residues K326, K328 and R147 were predicted to establish contacts with E181 of Tm. Moreover, K328 also potentially forms direct interactions with E286 of myosin when the motor is strongly bound. Post translational modifications (PTMs) alter the chemical nature of thin filament subunits. These modifications are widespread and play both physiological and pathological roles. Yet relative to the extensive study of disease-causing mutations, the impact of relatively few PTMs on contractile performance has been examined in the physiological context of muscle. Recently, LC-MS/MS analysis of the cardiac acetyl-lysine proteome revealed K326 and K328 of actin were acetylated; a reaction that masks the residues' inherent positive charge. Here, we tested the hypothesis that removing vital actin charges at residues 326 and 328 diminishes actomyosin interactions by altering Tm positioning and/or strong myosin binding, *in vivo*, as manifest by depressed skeletal and cardiac muscle function. Transgenic *Drosophila* lines were created that permit tissue-specific expression of K326Q, K328Q, or K326Q/K328Q acetyl-mimetic actin and of wild-type actin. Muscle-restricted transgene expression was confirmed with a GFP-based reporter. Compared to wild-type actin, expression of mutant actin had a dose-dependent effect on flight ability. Moreover, relative to K326Q actin, K328Q and K326Q/K328Q actin induced a more severe reduction in flight performance. High-speed video microscopy and motion analysis of beating hearts with cardiac-restricted transgene expression revealed significant reductions in systolic intervals among all acetyl-mimetic lines vs. control. Based on F-actin-Tm and F-actin-Tm-myosin models and on our physiological data, we conclude that acetylating K326 and K328 of actin alters electrostatic associations with Tm and/or myosin and thereby modifies actomyosin associations and muscle performance. Our findings highlight the utility of *Drosophila* as a model that permits efficient targeted design and assessment of molecular and tissue-specific responses to muscle protein modifications, *in vivo*.

P1664**Board Number: B176****Computational Modeling of Complex Mechano-Chemical Feedbacks in Acto-Myosin Networks.**G. Papoian¹, K. Popov¹, J. Komianos¹;¹Univ Maryland College Park, College Park, MD

Acto-myosin network growth and remodeling *in vivo* is based on a large number of chemical and mechanical processes, which are mutually coupled and spatially and temporally resolved. To investigate the fundamental principles behind the self-organization of these networks, we have developed detailed physico-chemical, stochastic models of actin filament growth dynamics, where the mechanical rigidity of filaments and their corresponding deformations under internally and externally generated forces are taken into account. Our work sheds light on the interplay between the chemical and mechanical processes, and also will highlight the importance of diffusional and active transport phenomena. For example, we showed that molecular transport plays an important role in determining the shapes of the commonly observed force-velocity curves. We also investigated the nonlinear mechano-chemical couplings between an acto-myosin network and an external deformable substrate.

P1665**Board Number: B177****Organization of actin cables in budding yeast: a computational model.**H. Tang¹, T.C. Bidone¹, D. Vavylonis¹;¹Department of Physics, Lehigh University, Bethlehem, PA

Budding yeast actin cables function as tracks for delivery of cellular organelles to the growing bud during polarized cell growth and aid in spindle orientation during mitosis. For proper cellular function, actin cables must form polarized bundles that grow from the bud to the mother, spanning the whole cell. Overexpression of Bni1 leads to increased density of actin cables and appearance of cable loops, while disruption of Bni1 leads to disappearance of actin cables. Regardless of the consistent body of experimental observations, what molecular mechanisms impact the large scale organization of actin cables in budding yeast remains elusive. Here, we develop a 3D computational model of actin cable dynamics to study the interplay between molecular mechanisms and large scale organization of actin cables in budding yeast. This model extends our prior study of actin cables in fission yeast (Tang et al. MBoC 2014). In this model, formins Bni1 and Bnr1 promote actin filament barbed end polymerization. The actin filaments are simulated as semiflexible polymers composed of beads connected by springs. Formin turnover is incorporated as filament detachment and recruitment at cortical sites. Cross-linking by fimbrin Sac6 is simulated as a short-range attractive interaction. Pulling by class V myosin and class II myosin are simulated as tangential forces on filament beads. Cofilin-induced severing is implemented as breaking and disassembly of filament segments. We show that with these mechanisms, the model can generate the actin cable structures similar to those observed in experiments. We use this model to study

what is the origin of the transition between straight cables and cables forming loops as a function of different combinations of the molecular mechanisms and to identify patterns of forces leading to cable buckling. These simulations illustrate how biophysical and biochemical properties combine to establish different types of cytoskeletal organization at the cellular scale.

P1666

Board Number: B178

Forces driving epithelial wound healing.

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The rapid restoration of epithelial integrity after wounding is a crucial process for the survival of complex organisms. Epithelial wound healing is commonly attributed to a combination of two mechanisms. The first one is the so-called purse-string contraction, whereby cells lining the wound assemble and contract a supracellular actomyosin cable. The second mechanism is cell crawling driven by lamellipodial and filopodial protrusions. The relative contribution of these two mechanisms to wound healing remains controversial, however. Here we combined laser ablation, traction force microscopy and micropillar force detectors to measure the first complete maps of the mechanical forces driving wound healing. These maps demonstrate that migration is driven initially by the cooperative action of multiple cell rows crawling into the wound. At later stages, we observed traction forces with unanticipated components radial and tangential to the wound boundary. We show that these force components arise from tensions in a heterogeneous actomyosin ring and that these tensions are transmitted to the underlying substrate through focal adhesions. A computational model we developed captures tractions and tensions in the monolayer and their relationship to cellular shapes and motions. These findings demonstrate that the actomyosin ring is a much more versatile structure than previously thought and serves at least two purposes. First, it contributes to wound closure through its well-known purse-string mechanism. Second, it contracts heterogeneously to compress the underlying substrate.

P1667**Board Number: B179****Spatial organization of the cellular actin cortex during the cell cycle.**P. Chugh¹, A.G. Clark¹, M.B. Smith¹, K. Dierkes², G. Salbreux³, E.K. Paluch¹;¹MRC-LMCB, UCL, London, United Kingdom, ²Centre for Genomic Regulation (CRG) and UPF, Barcelona, Spain, ³MPI PKS, Dresden, Germany

Cell shape regulation is key to a number of fundamental biological processes, including cell migration and division. In animal cells, cell morphology is controlled primarily by the cortex, a thin actomyosin network underlying the plasma membrane. The cortex determines global physical properties of the cell such as tension and stiffness. Previous studies have shown that spatial and temporal changes in cortical tension drive shape changes during the cell cycle such as mitotic rounding and cytokinetic furrow ingression. However, the precise changes in cortical structure and composition required to generate changes in cortical tension during the cell cycle remain unclear. We are investigating this question using a combination of cell biology experiments, quantitative imaging and modeling. As the cortex dimensions are below the resolution limit of conventional light microscopy, we have developed a dual-color localization method to investigate the spatial organization of the cortex. This method is based on estimating the relative localization of cortex components with respect to one another by labeling them with chromatically different fluorophores. Using our method, we quantified cortex thickness and compared the localization of key actin binding proteins in different stages of the cell cycle. We could show that cortex thickness is lower in prometaphase than in interphase. Interestingly, we also observed that myosin motors differentially localize in the cortex during interphase as compared to mitosis. We are currently investigating the factors that control cortex thickness as well as the localization of myosin and other key cortical components during the cell cycle. Combined with a physical model of the cortex, our systematic analysis will help uncover the mechanism by which cortical structure and organization regulate cortical mechanics, thereby driving cellular morphogenesis.

P1668**Board Number: B180****Spatial organization of nuclear membrane cytoskeleton.**J. Usukura¹;¹Structural Biology Center, Nagoya University, Nagoya, Japan

The spatial organization of nuclear cytoskeleton was investigated at high resolution using simultaneous imaging of cryo-bright field, dark field STEM and SEM, and also conventional immune-freeze-etching methods. Nuclear cytoskeleton showed quite different spatial structure from plasma membrane cytoskeleton, though it consisted of actin filaments, microtubules and intermediate filaments. Nuclear surface was covered with thick layer of intermediate filaments (Vimentin in NRK cells), while many actin filaments extend from the nuclear membrane and form stress fibers similar to the plasma membrane cytoskeleton. However, they never organize a meshwork by branching with Arp 2/3 that is commonly

seen in cortical area of cell membrane. Instead, they are intimately associated with intermediate filaments. In particular, at the surface of nucleus, intermediate filaments surrounded actin filaments like a sheath, which extended from the periphery of nuclear pores forming complicated meshwork and covered the total surface (depending on unroofed cryo-SEM images). Actin filaments were secondary major components in nuclear cytoskeleton. We investigated polarity of actin filaments by S1 decoration method. Both pointed and barbed ends were detected on the surface of nucleus, that is, both origin and termination of actin filaments present on the nuclear membrane. Probable candidate of actin binding site has been considered to be Nesprin 1 and 2 so far. Indeed, they were found abundantly on the nuclear surface in immuno-labeling. Unfortunately, however, we were not able to observe directly the binding between actin filaments and Nesprins, because actin filaments were covered with intermediate filaments on closing the nuclear surface. Under careful observation, some actin filaments appeared to extend from nuclear pores, judging from EM photographs.

P1669

Board Number: B181

Probing *in vivo* dynamics of mitochondria and cortical actin networks using high-speed atomic force/fluorescence microscopy.

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Dynamics of the cell membrane and submembrane structures are closely linked to achieve various cellular activities. Although cell surface research and cortical actin studies have revealed their mechanisms of action independently, it has been difficult to obtain a comprehensive understanding of the dynamics of these structures in live cells. Here we utilized a high-speed atomic force microscopy combined with an optical microscopy system to analyze membrane-based cellular events at nano-scale resolution in live cells. Imaging the COS-7 cell surface revealed detailed structural properties of membrane invagination events corresponding to endocytosis and exocytosis. In addition, the movement of mitochondria and the spatiotemporal dynamics of cortical F-actin network were directly visualized *in vivo*. Cortical actin microdomains with an average size of 231 ± 69 nm were dynamically rearranged by newly appearing actin filaments, which sometimes accompanied membrane invaginations, suggesting that these events are integrated with the dynamic regulation of submembrane organizations maintained by actin turnovers. These results provide novel insights into the structural aspects of the entire cell membrane machinery which can be visualized with high temporal and spatial resolution.

P1670**Board Number: B182****Formins are essential contributors to the assembly and maintenance of *Drosophila* sarcomeric thin filament arrays.**A. Shwartz¹, E.D. Schejter¹, B. Shilo¹;¹Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel

Structural organization of striated skeletal myofibers, and of the repeating sarcomere units from which they are composed, is critical for efficient performance of muscle motor functions. Our study focuses on assembly of the sarcomeric actin-based thin filament array and its subsequent maintenance, using the sarcomeres of *Drosophila* indirect flight muscle (IFMs) as our model system. Employing GFP-tagged G-actin incorporation as a tool to monitor the dynamic assembly of microfilaments, we have been able to follow the events underlying thin-filament array generation and provide a timeline for their progression. This sequence includes bundling of pre-existing filaments, nascent array elongation, and array “thickening” via addition of peripheral filaments. Monitoring of array dynamics in adult flies suggests that maintenance via filament turnover occurs primarily at the barbed (Z-disc associated) and pointed ends of the arrays. Using genetic approaches we have identified a variety of functional requirements for the formins Fhos/FHOD and Form3/INF2 in establishment and maturation of the IFM thin-filament arrays. Fhos in particular appears to play several key roles during the different phases of array assembly and growth, as well as contributing to maintenance of mature arrays. Current studies are aimed at validating a model by which these formins use their capping, bundling and elongation capacities to perform their different roles.

P1671**Board Number: B183****Arp2/3 complex-dependent assembly of filopodia by the formin FMNL3 contributes to cell-cell adhesion.**L.E. Young¹, T.J. Gauvin², E.G. Heimsath³, H. Higgs⁴;¹Dartmouth College, Hanover, NH, ²Dartmouth-Geisel Sch Med, Hanover, NH, ³NIH/NIDCD, Bethesda, MD, ⁴Department of Biochemistry, Geisel School of Medicine at Dartmouth College, Hanover, NH

Filopodia are finger-like, actin-dependent membrane protrusions that play fundamental roles in directional cell migration, cell-cell adhesion and chemosensing. The mechanism for filopodial assembly remains to be established, with two predominant mutually exclusive models: 1) the tip nucleation model describes that filopodia are created de novo by a molecule that nucleates new actin filaments; or 2) the convergent model describes that filopodia are re-modeled from Arp2/3 complex-nucleated dendritic networks, such as lamellipodia. Previously, we have shown that two formin proteins, FMNL3 and mDia2, are potent filopodial inducers, but whether they act by the tip nucleation or convergent model was uncertain. Here, we show that both FMNL3- and mDia2-induced filopodia are largely Arp2/3 complex-

dependent in multiple cell types (non-adherent and adherent). Interestingly, a small percentage of these filopodia are Arp2/3 complex-independent, suggesting that FMNL3 and mDia2 may use actin filaments generated by alternative nucleators in certain situations. Biochemical assays show that, under conditions that mimic mammalian cytosol, both FMNL3 and mDia2 are poor nucleators, further suggesting the need of a separate nucleation factor during filopodial assembly. To address the physiological function of FMNL3, we show that the full-length protein (both endogenous and GFP-tagged proteins) localizes predominately at the plasma membrane, where it enriches in filopodia, membrane ruffles, and at cell-cell contact sites. Interestingly, FMNL3-enriched filopodia are 10-fold more stable when at the cell-cell interface in comparison to when interacting with the cell-free coverslip surface. A small proportion of FMNL3 localizes to dynamic vesicular structures in the cytoplasm; and these structures can migrate to and fuse with the plasma membrane at cell-cell contact sites. Suppression of FMNL3 causes defects in filopodial assembly and cell-cell adhesion. To summarize, our results show that FMNL3 and mDia2 assemble filopodia in an Arp2/3 complex-dependent manner. FMNL3-dependent filopodia in particular are specialized for cell-cell adhesion.

P1672

Board Number: B184

Colocalization of RhoGAP restricts the size of pulsatile actomyosin foci in *C. elegans* embryos.

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Cortical actomyosin often forms pulsatile foci during tissue morphogenesis. How the foci structure is organized is under intense study. Pulsatile actomyosin foci are also found in one-cell stage embryos of *C. elegans*. Although numerous foci cover the cell surface, these foci are largely uniform in size, suggesting size regulation. However, it is unknown how the size of myosin foci is regulated.

Here we show that a RhoGAP protein RGA-3 colocalizes with the myosin foci and this colocalization regulates the size of myosin foci. We observed that GFP::RGA-3 colocalizes with a mCherry fusion of myosin regulatory light chain MLC-4. In order to investigate the function of this colocalization, we expressed a truncated form of RGA-3 that localizes to cytoplasm in *rga-3/4(RNAi)* background. This cytoplasmic RhoGAP is functional because it rescued the hypercontractile phenotype of *rga-3/4*. However, there was a marked difference in the cortical myosin organization. Myosin in wild-type embryos forms multiple foci, whereas myosin in the cytoplasmic RhoGAP embryos formed a large ring-like structure. This difference is not due to the expression level of RhoGAP because neither partial depletion nor overexpression did not cause the ring formation.

Next, we fused an F-actin binding domain to the cytoplasmic RhoGAP. When this actin-binding RhoGAP was expressed, cortical myosin formed multiple foci similarly to wild type, indicating that the colocalization of RhoGAP and actomyosin is sufficient to prevent the formation of the large ring. Based

on these observations, we propose the following model. The colocalization of RhoGAP to myosin foci constitutes a negative feedback, which restricts the size of myosin foci by preventing overgrowth.

P1673

Board Number: B185

Septin complexes assemble end-over-end in cells.

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The septins are an essential family of filament-forming GTP-binding proteins with conserved functions in cell division. Yeast septins form octameric, nonpolar, rod-shaped complexes of about 32 nm length and assemble further into higher-order structures that perform a variety of functions in the cell cycle. While in vitro the assembly of complexes into filaments is quite well understood, how the complexes assemble into the higher-order structures in cells remains unclear. Here, we used single molecule localization microscopy to visualize both termini of septin rods at nanometer resolution in vitro and in cells. Single septin complexes appeared as pairs of localizations around 30 - 35 nm apart and revealed the exact spatial orientation of the complex in space. Under in vitro conditions favorable to septin polymerization, we detected septin assemblies as very thin, elongated stretches of equidistant localizations both when Cdc11, the terminal subunit of the rod, and when Cdc10, the central subunit of the rod, was labeled and detected. These filaments were mostly straight and occasionally appeared bundled. In a filamentous fungus, we resolved similar localization pairs and thin filaments of equidistant localizations. Our work demonstrates that septin complexes assemble end-over-end into filaments in cells and that if paired, filaments are aligned in register.

P1674

Board Number: B186

Long cytoplasmic projections (“cytonemes”) in antral follicles in mouse ovary.

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We used serial section electron microscopy to examine mouse ovarian antral follicles. The granulosa cells around the follicle periphery and the cumulus cells surrounding the oocyte are in constant communication with each other and with the oocyte. We found long cytoplasmic projections (“cytonemes”) from granulosa and cumulus cells that are likely to have an important role in cell-cell

interactions. Similar filipodia-like projections emanate from sea urchin blastomeres, *Drosophila* cells, and lymphocytes in culture; they have also been called 'nanotubes', or 'thin projections'. In *Drosophila*, there is evidence that they take part in decapentaplegic signaling. The serial section electron microscopy was done using a new method developed by Lichtman and collaborators. Sections are cut on a conventional ultramicrotome, collected on tape using an "ATUM" instrument, and examined by field emission scanning electron microscopy. The sections are collected more reliably than with conventional methods. Furthermore, large sections (2 x 4 mm) are collected, which increases the area that can be searched. For this study, approximately 100 sections of 60 nm thickness were examined. In preliminary observations, there are ~6-10 cytonemes per cell. They have a diameter of ~150 nm, are between 1 and 2 cell diameters in length (5-10 um), and appear to have no preferred directional orientation within the follicle. They sometimes form bundles, and touch or make invaginations into neighboring cells without any apparent specialization at the end. The zona pellucida is a thick acellular region surrounding the oocyte. Long filamentous projections, called trans-zonal processes, originate from cumulus cells and cross the zona pellucida. At the oocyte, the ends of these processes form a specialized structure which are the sites of gap junctions between cumulus cells and the oocyte. We suggest that cytonemes have cell to cell functions between cumulus / granulosa cells, but they also can traverse the zona pellucida and become induced to form a trans-zonal process when they contact the oocyte.

Actin-Membrane Interactions

P1675

Board Number: B187

Intersectin-1 mediates recruitment of AP-2 and clathrin to Vaccinia virus.

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Immediately following fusion with the plasma membrane, Vaccinia virus recruits clathrin in an AP-2 dependent fashion before undergoing actin-based motility. The recruitment of clathrin helps polarize the integral viral membrane protein, A36 beneath the virus. Polarization of A36 in turn leads to clustering of N-WASP enhancing Arp2/3 complex dependent actin polymerisation to promote viral spread.

We now sought to uncover the molecular basis by which Vaccinia recruits clathrin via its adaptor protein AP-2. Using a combination of approaches we have uncovered that AP-2 and clathrin recruitment is dependent on three NPF (Asn-Pro-Phe) motifs in the C-terminus of A36. These NPF motifs mediate clathrin recruitment by interacting with the Epsin15 Homology (EH) domains of intersectin-1. A recombinant A36 Vaccinia virus lacking all three NPF motifs is deficient in intersectin-1, AP-2 and clathrin recruitment. This results in a delay in the initiation of actin polymerization and a decrease in viral spread

that is reminiscent to that seen in the absence of clathrin.

To our knowledge A36 represents the first viral protein containing NPF motifs that interacts with endocytic machinery. The three NPF motifs are highly conserved in A36 homologues in other Orthopoxviruses, suggesting they provide a selective advantage for viral spread. The presence of the NPF motifs is also predicted to aid recycling of viral proteins from the plasma membrane to the TGN for additional cycles of viral assembly. Future studies will examine if this is the case and the role of other endocytic components in viral assembly.

P1676

Board Number: B188

Plasma Membrane Tension Triggers Adhesion Positioning in the Leading Edge of Cells.

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Cell migration is dependent on adhesion deposition and actin cytoskeleton remodeling at the leading edge. These events may be physically constrained by the plasma membrane. Here, we report that the mechanical signal produced by an increase in plasma membrane tension triggers the positioning of new rows of adhesions at the leading edge. During lamellipodial protrusion, as membrane tension increases, the protrusion velocity slows, and the polymerizing actin buckles upward in a myosin II independent manner. The buckling occurs between the front of the lamellipodium, where nascent adhesions are deposited in rows (containing integrin, paxillin, vinculin, talin, vasp and zyxin) and the base of the lamellipodium, where a vinculin-dependent clutch couples actin to previously deposited adhesions. As membrane tension decreases, protrusion resumes and buckling disappears until the next cycle. We propose that the mechanical signal of membrane tension exerts an upstream control in mechanotransduction by periodically compressing-relaxing the lamellipodium.

P1677

Board Number: B189

Role of actin filaments in mobility of connexin 36 in HeLa cells.

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Electrical synapses formed of the gap junction (GJ) protein Cx36 exhibit a high level of functional plasticity. Changes in connexin phosphorylation alter channel permeability on a minutes time scale,

while connexin turnover may alter channel abundance on longer time scales of minutes to hours. In order to better understand mechanisms of turnover we studied the role of cytoskeletal elements, actin filaments in particular, in Cx36 vesicle trafficking and GJ mobility. We utilized HaloTag technology with fixed and live-cell imaging to track the movement of Cx36 GJ plaques and vesicles in transiently transfected HeLa cells. The HaloTag protein forms irreversible covalent bonds with chloroalkane ligands, allowing specific protein labeling. The HaloTag open reading frame was inserted into an internal site in the C-terminus of Cx36 designed not to disrupt regulatory phosphorylation sites or C-terminal protein-protein interactions of Cx36. Pulse-chase labeling with fluorescent HaloTag ligands allowed clear discrimination of newly formed and pre-existing Cx36. Cx36-Halo formed large junctional plaques at sites of contact between transfected HeLa cells and was also contained in many intracellular vesicles. Phalloidin labeling showed that thick actin bundles connected all edges of GJ plaques, but actin filaments were rare within any plaque. Actin filaments were also found associated with small, chase-labeled delivery vesicles. Many GJs showed substantial numbers of finger-like filadendrites extending from both the edges and the center of the plaques, and the morphology of these filadendrites changed at a fast pace. These filadendrites consisted solely of pulse labeled Cx36, and their morphology was constantly rearranged by breaking off and fusing back with the plaques. Double labeling of HaloTag ligand and phalloidin showed that these filadendrites colocalized with thin actin filaments. Disruption of actin filaments with Cytochalasin D by binding to F-actin polymers caused loss of GJ at cell-cell contacts. Treatment with Latrunculin A, which binds to actin monomers and prevents polymerization and filament elongation, did not disrupt GJ plaques and only partially suppressed Cx36 turnover, but eliminated the filadendrite extensions. We conclude that actin bundles are critically important for the stabilization of Cx36 GJ plaques and that actin filaments are involved in the dynamic mobility of these plaques. The role of rapid mobility of elements of GJ plaques in functional plasticity is unknown, but we hypothesize that it is related to the mechanisms that control turnover of connexin protein.

P1678

Board Number: B190

Novel roles for cholesterol and the cytoskeleton in the formation of neutrophil extracellular traps.

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Background/Purpose: The role of neutrophils in immunity has drawn increasing attention over the last decade. A recently discovered structure made by these cells, neutrophil extracellular traps (NET), form from disintegration of the nuclear envelope and mobilization of nuclear material from the nucleus to the extracellular milieu. NETs have been found to be important in infectious, autoimmune and cardiovascular diseases. Recent articles reported the presence of NETs in atherosclerotic lesions, and NETs promote thrombosis. Blockade of peptidylarginine deiminase, an enzyme that mediates chromatin decondensation and NET formation, attenuates atherosclerosis burden and arterial thrombosis in mice.

Hypercholesterolemia is a primary risk factor for athero-thrombotic cardiovascular diseases. However, the effect of cholesterol loading on NET formation and the relevant cellular mechanisms have not been investigated.

Methods: Primary human neutrophils were isolated from the blood of healthy donors by sequential centrifugation with Histopaque 1077 and 1119. Exogenous cholesterol was delivered to these cells as a water-soluble complex with methyl- β -cyclodextrin (MCD, Chol/MCD), which has been widely used to modify the cholesterol content of cultured cells without potentially confounding effects from receptor engagement. Incubations lasted for up to 6 hours, followed by fixation with 2% PFA and then Sytox Green staining for NET formation. In some experiments, neutrophils were pretreated with inhibitors of myosin II ATPase (blebbistatin) or myosin light chain kinase (ML7), or with the inhibitors of actin (Cytochalain D) or microtubule (Cochicine and XRP44) polymerization.

Results: We found that cholesterol loading with Chol/MCD induced NET formation in a dose-dependent manner in primary neutrophils, as quantified by a fluorescent microplate reader and the NET structure confirmed with fluorescent microscopy. Inhibition of myosin II ATPase or myosin light chain kinase, or inhibition of actin or microtubule polymerization by pretreatment of neutrophils with their inhibitors significantly attenuated cholesterol-induced NETosis in vitro.

Conclusion: Our studies indicated that cholesterol loading induces neutrophil extracellular trap formation in vitro. Inhibition of actin and microtubule cytoskeletal machinery attenuates cholesterol loading induced NETosis. Our findings may provide a mechanistic basis for the role of neutrophils and NETs in complications of hypercholesterolemia.

P1679

Board Number: B191

Cholesterol depletion disrupts myofibril organization and changes mechanical properties of cardiac myocytes.

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Cholesterol regulation plays a crucial role in our physiology, with high levels resulting in formation of atherosclerotic plaques, stroke and cardiovascular diseases. Cholesterol lowering drugs, such as statins, are ubiquitous means to treat these diseases by inhibiting HMG-CoA reductase, which regulates cholesterol synthesis. Recently, statins are also being used to prevent cardiovascular diseases in individuals with normal cholesterol levels which might cause a drastic diminishment in their cholesterol levels. The consequences of reduced levels of cholesterol on cell physiology have not been well

explored, specifically; the effects on cardiac cells. In this work we have studied the effects of cholesterol depletion in neonatal rat cardiomyocytes using M β CD, a cyclodextrin that depletes cholesterol from the plasma membrane, and H γ CD, a cyclodextrin that has low affinity for cholesterol, as a control. By using immunofluorescence we observed that cholesterol depletion impacted the organization of striated myofibrils, changing the density and distribution of α -actinin bands. To assay the mechanical consequences of this altered actin cytoskeleton, we examined the contractility of myocytes and we found that cholesterol depletion increased the rate and variability of cell contraction. We also measured changes in the cortex mechanics by pulling tethers using optical tweezers and found that cholesterol depletion increased both the surface tension and bending modulus in those cells. Overall, these results bring the attention to possible side effects of cholesterol lowering therapies in normal individuals.

P1680

Board Number: B192

Membrane retrieval coincides with the filopodia formation in the neuronal growth cone.

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The neuronal growth cone is a motile tip of the growing neurite at the times of the neuronal development and the regeneration. At the leading edge of the growth cone, the continuous rearrangement of actin cytoskeletons, and the membrane retrieval from the plasma membrane, are observed. These two events should be essential to the accurate navigation of neurite, however, the relationship between them is not clearly understood. To visualize the vesicular trafficking and actin reorganization of the growth cone in detail simultaneously, we analyzed the dynamics of GFP-synaptophysin and mCherry-actin in the growth cone of NG108-15 cells, using a superresolution microscopy SR-SIM. We found that the vesicles arose near the filopodia, and most of them were retrogradely moving along the actin bundles. When actin polymerization inhibited by latrunculin B, such vesicles accumulated at the leading edge, accompanied with disappearance of the actin bundles. RNAi of fascin also decreased the numbers of vesicles localized at the leading edge. Moreover, Eps15, a component of clathrin-mediated or -independent endocytosis, was localized along the actin bundles. These results suggest that there is a novel mechanism of membrane retrieval in the growth cone; the vesicle production by endocytosis occurring at the leading edge, coincides with filopodia formation and these vesicles are moving along the retrograde flow of the actin.

P1681**Board Number: B193****Mechanochemical model of cell blebbing with actin cortex healing allows elucidation of traveling bleb dynamics.**K. Scannell^{1,2}, J.F. Allard^{1,2,3};¹Dept. of Mathematics, University of California, Irvine, Irvine, CA, ²Center for Complex Biological Systems, University of California, Irvine, Irvine, CA, ³Dept. of Physics and Astronomy, University of California, Irvine, Irvine, CA

Blebs are pressure-driven cell protrusions implicated in cellular functions such as cell division, apoptosis, and cell motility including motility of protease-inhibited cancer cells. Because of their mechanical nature, blebs can inform us about general cell mechanics including membrane dynamics, pressure propagation throughout the cytoplasm, and the architecture and dynamics of the actin cortex. Mathematical models including detailed fluid dynamics have previously been used to understand bleb expansion. Here we develop mathematical models that recapitulate the full bleb life cycle, including both expansion and healing by cortex reformation. The model can be reduced to a set coupled partial differential equations with a non-diffusion-like spatial operator, amenable to two-component phase-plane analysis. The model provides conditions under which blebbing occurs, and predicts how bleb size and shape vary with experimentally-accessible biophysical parameters such as myosin contractility, osmotic pressure, and turnover of actin and ezrin. The model also exhibits traveling blebs that move along the cell periphery. We predict the conditions under which blebs travel or remain stationary, and predict the bleb traveling velocity, a quantity that has remained elusive in previous models.

P1682**Board Number: B194****MyoGEF localizes to the bleb membrane and promotes bleb retraction.**M. Jiao¹, D. Wu¹, S. Zu¹, C.C. Sollecito¹, A.J. Mold¹, K. Jimenez-Cowell¹, Q. Wei¹;¹Department of Biological Sciences, Fordham Univ, Bronx, NY

Membrane blebbing has been implicated in cytokinesis, cell migration, and apoptosis. The life cycle of a bleb consists of initiation, expansion, and retraction. Following bleb initiation and expansion, re-assembly of a contractile actomyosin network underneath the bleb membrane results in bleb retraction. RhoA/Rho-kinase (ROCK) signaling can stimulate the formation of the contractile actomyosin cytoskeleton. Rho GTPase proteins such as RhoA are primarily activated by guanine nucleotide factors (GEFs). It is generally thought that RhoA can promote bleb retraction by driving the re-assembly of a contractile actomyosin network underneath the bleb membrane. However, it is still unclear how GEFs are implicated in promoting bleb retraction through activation of RhoA at the bleb membrane. MyoGEF is a GEF that can promote cytokinesis and breast cancer cell invasion through activation of RhoA. Here we report that MyoGEF is implicated in the regulation of membrane blebbing. Live cell imaging analysis shows that MyoGEF colocalizes with ezrin, a membrane-cortex linker, to the bleb membrane during late

stages of bleb expansion. More importantly, the arrival of ezrin and MyoGEF at the bleb membrane is coincident with bleb retraction. We propose that the ezrin-MyoGEF interaction results in the recruitments of MyoGEF to the bleb membrane, where MyoGEF activates RhoA and promotes the assembly of a contractile actomyosin network, leading to bleb retraction.

P1683

Board Number: B195

Re-organization of the local membrane environment upon Integrin engagement.

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The plasma membrane of the living cell acts as an interface between the cell and its external milieu. It serves as a dynamic conduit for information transduction and response. It has been proposed that the regulated lateral segregation of plasma membrane associated molecules into distinct domains help in coordinating various signal transduction functions. Previous work from our laboratory and others has shown that proteins such as the outer leaflet GPI-anchored proteins, the inner leaflet Ras proteins and several glycolipids form dynamically reorganizing domains consisting of nanometer sized clusters^[1]. These passive molecular arrangements are a consequence of the local dynamics and organization of cortical actin filaments^[2]. In contrast, we had proposed that signaling receptors such as the Integrin receptors actively regulate the composition of the membrane in their vicinity by controlling the actin dynamics. Integrins represents a family of hetero-dimeric trans-membrane receptors that link extracellular ligands to a multitude of signaling and structural molecules^[3]. Although previous experiments have reported that the zones of integrin activation are enriched in lipid-raft like elements^[4,5], the molecular mechanism as well as the physiological relevance of creating such domains has not been explored.

Here we present our understanding of the molecular machinery that regulates the dynamics of cortical actin filaments, which in turn facilitate the generation of localized actin asters that leads to the active focusing of cell surface molecules. Using the technique of homo-FRET based fluorescence emission anisotropy, we report that the engagement of integrins that bind the extracellular matrix ligand fibronectin, results in the enrichment of GPI-anchored proteins clusters in the vicinity of the activated receptor. Our results suggest that Integrin receptors may fine-tune membrane organization by exploiting the mechanism regulating the dynamic cortical actin filaments, thereby facilitating the creation of localized domains. The subsequent recruitment of molecules that have an affinity for these domains is likely to play an important role in the regulation of Integrin function itself.

REFERENCES:

- 1.) Rao and Mayor, Active organization of membrane constituents in living cells, *Current Opinion in Cell Biology*, 2014
- 2.) Gowrishankar et al, Active Remodeling of Cortical Actin Regulates Spatiotemporal Organization of Cell Surface Molecules, *Cell*, 2012

- 3.) Hynes RO, Integrins: bidirectional, allosteric signaling machines, *Cell*, 2002
- 4.) van Zanten et al, Hotspots of GPI-anchored proteins and integrin nanoclusters function as nucleation sites for cell adhesion, *PNAS*, 2009
- 5.) Gaus et al, Integrin-mediated adhesion regulates membrane order, *JCB*, 2006

P1684

Board Number: B200

Role of Bar-domain containing protein Syndapin in remodeling actin in the early *Drosophila* embryo.

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Rapid remodeling of the plasma membrane (PM) and actin cytoskeleton is crucial during the syncytial stages of development in the *Drosophila* embryo where rapid and concerted nuclear divisions occur in mitotic waves across the length of the embryo. Syndapin has an F-Bar domain that can induce membrane curvature as well as SH3 domain that is known to interact with actin-modulating proteins N-Wasp and Anillin and is thus poised to orchestrate concerted membrane and actin remodeling. We have investigated the role of Syndapin in these syncytial stages of *Drosophila* embryo development. Like Actin, Syndapin is localized along both the apical and lateral membranes during the syncytial nuclear cycles, with a distinct enrichment at the tip or furrow canal. Syndapin mutant embryos show depletion of both Syndapin and Actin levels. This has distinct morphological effects during the syncytial cycle – defects in separation of nuclei and apical caps and shorter membrane furrows are seen. These defects could be in part due to a lowering and mislocalization of cytokinetic proteins Anillin and a member of the septin family, Peanut. We examined the distribution of Syndapin in Anillin and Peanut mutant embryos to test for their interdependence for associating with the PM. Anillin mutant embryos showed both Peanut and Syndapin mislocalization from the membrane. Peanut mutant embryos, however, showed only partial Anillin mislocalization and normal Syndapin. These data together confirm the requirement of Anillin and Syndapin on the PM for proper association of Peanut to give rise to concerted actin cap and membrane remodeling during the syncytial cycles. Actin cap morphology was further assessed in detail using live imaging with lifeact-GFP using Confocal and TIFR microscopy. Actin protrusions were found to remain longer in Syndapin mutant embryos during the syncytial cycles as compared to wildtype where the size of protrusions was relatively longer in interphase. There were also a greater number of protrusions with greater residence time, further illustrating poor actin remodeling. These defects could be explained due to a mislocalization of Arp2/3 on the PM. While fluorescently-tagged actin-associated proteins marked protrusions on the PM surface, Syndapin-GFP showed a remarkable punctate pattern, suggesting localization to the tip of the protrusion on the PM cortex. Taken together, our data shows that Syndapin recruitment along with Anillin and Arp2/3 is required for actin remodeling on the apical membrane and in furrows in metaphase to maintain membrane and actin dynamics in the early *Drosophila* embryo.

P1685**Board Number: B201****Contact Localized β -actin Translation Drives Epithelial Adherens Junction Assembly and Establishes Tissue Barrier Permeability.**N. Gutierrez¹, A.J. Rodriguez¹;¹Federated Department of Biology, Rutgers University, Newark, NJ

Adherens junctions are multi-protein adhesion complexes that anchor to the actin cytoskeleton at cell-cell contact sites to stimulate tissue assembly and thereby regulate embryonic development, barrier maintenance, and epithelial tissue homeostasis. Previously it was demonstrated that the mRNA for β -actin contains a nucleotide sequence in its 3' UTR, called the zipcode, which is necessary for targeting and translational regulation of this transcript. The zipcode sequence is recognized by the translation regulator zipcode binding protein-1, ZBP1. Additionally, deleting zipcode sequences causes mislocalization of their mRNA transcript. Here we show mislocalizing β -actin's monomer synthesis inhibits adherens junction assembly following epithelial cell-cell contact. Additionally, inhibiting RhoA and Src signaling, required for β -actin mRNA zipcode-mediated spatially regulated β -actin translation, prevents adherens junction assembly and significantly perturbs epithelial barrier integrity. In fact, barrier permeability significantly increases in epithelial cells expressing β -actin transcripts without the 3' untranslated region (Δ 3'UTR- β -actin) during TransWell tissue permeability experiments. Moreover, in full-length β -actin GFP (FL- β -actin GFP) expressing cells, β -actin mRNA zipcode antisense oligonucleotide masking of the zipcode sequence also perturbs barrier maintenance. Intriguingly, chemically inhibiting the signaling pathways required for β -actin mRNA localization and local translation (RhoA and Src) also perturbs epithelial barrier maintenance. Taken together we demonstrate epithelial cell-cell contact stimulates E-cadherin mediated signaling to spatially regulate β -actin mRNA zipcode-dependent monomer synthesis thereby driving adherens junction assembly and tissue adhesion required for proper epithelial tissue function.

P1686**Board Number: B202****Septins are essential for tissue integrity during epithelial morphogenesis.**M. Mavrikis¹, Y. Azou-Gros¹, A. Kress², S. Brasselet², T. Lecuit¹;¹Institut de Biologie du Développement de Marseille, CNRS UMR 7288, Marseille, France, ²Institut Fresnel, CNRS UMR 7249, Marseille, France

Septins are highly conserved cytoskeletal proteins that have been mostly studied in dividing cells. Besides their role in cytokinesis, recent studies suggest that septins might regulate the organization of the actin cortex in nondividing cells. However, the precise contribution of septins to the architecture of the cortex in nondividing cells is still unknown, and the molecular functions of septins altogether are poorly understood. To test the role of septins in actin-driven cell shape changes *in vivo*, we studied

epithelial tissue morphogenesis using the gastrulating *Drosophila* embryo as a model system. To study the architecture of the actin cortex and the overall organization of the epithelial tissue during morphogenetic movements, we used a combination of live two-photon imaging, transmission and scanning electron microscopy and polarization-resolved fluorescence microscopy in wild-type and septin-deficient whole embryos. Importantly, septins appear to have a major role in maintaining the integrity of the remodeling epithelial tissue. In the absence of septins, epithelial polarity is lost, the bases of epithelial cells open up, cell membranes undergo excessive blebbing and are eventually fragmented leading to the complete loss of cell integrity and the collapse of the epithelial tissue within an hour after the onset of gastrulation. We will discuss these findings in the context of our recent report on the capacity of septins to cross-link and organize actin filaments in contractile rings (Mavrakakis et al, 2014).

P1687

Board Number: B203

Actomyosin drives membrane dynamics in an in vitro active composite layer.

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The surface of a living cell provides a platform for processes such as receptor signaling, protein sorting, transport and endocytosis. The regulation of these processes requires the controllable organisation of membrane components. A recent framework for the organisation of a certain class of plasma membrane components is based on the active mechanics of acto-myosin juxtaposed to the membrane (Gowrishankar et al., 2012; Rao & Mayor, 2014). A systematic study of the dynamics and consequences of this active composite in living cells is challenging. Here we reconstitute an active composite in vitro, by a stepwise addition of the minimal ingredients: a supported lipid bilayer with an actin-binding component, short actin filaments and myosin motors. By systematically varying the concentrations of actin and myosin as well as the level of ATP we find a rich phase diagram of membrane-confined actin and myosin configurations. By increasing the level of available ATP we induce a constitutively remodeling state, in which asters composed of short actin filaments form and dissolve. In this state, the coupling of actin to the bilayer drives the membrane components out of equilibrium, imparting distinct signatures of activity in a manner entirely consistent with measurements in the living cell. These results highlight the fundamental basis of the active composite framework and indicate its relevance in the study of membrane organisation.

P1688**Board Number: B204****Microvillar tip-localized interaction between IRSp53 and PI(4,5)P₂ drives brush border assembly in kidney epithelial cells.**S. Kurisu¹, T. Itoh¹, T. Takenawa¹;¹Kobe Univ. Biosignal Research Center, Kobe, Japan

Kidney epithelial cells lining the proximal tubule develop a highly ordered, closely packed array of actin-based protrusions, known as brush border microvilli. Despite their importance in renal resorption, little is known about the shape regulation of membrane-cytoskeleton organization. Initially, we found that IRSp53, a member of the I-BAR family proteins, is concentrated at the tip of brush border microvilli both in the proximal tubule of mouse kidney and in cultured cells. Depletion of IRSp53 resulted in severe impairment of brush border formation. Each microvillus became short and dynamic in contrast to an ordered array of static microvilli of control cells, indicating a role for IRSp53 in converting short individual microvilli into a bunch of long microvilli forming brush border. Using stable cell lines that express several different GFP-tagged PH domains that recognize a specific phosphoinositide species, we show that IRSp53 induces brush border formation by binding to PI(4,5)P₂ at the tip of microvilli. As reported previously, the distal part of microvilli is enriched with PLCδ1-PH (δ1PH) domain, a PI(4,5)P₂ sensor. Notably, depletion of IRSp53 results in separation of PI(4,5)P₂-enriched membrane microdomains from microvillar F-actin bundles in the apical membrane. Expression of IRSp53 I-BAR domain mutants that are defective in phosphoinositide-binding show similar effects, indicating a possible role for the I-BAR domain in clustering PI(4,5)P₂ at the growing ends of microvilli. Surprisingly, a fusion protein consisting of δ1PH domain fused to IRSp53 I-BAR mutant induces ectopic brush border formation in basolateral membranes, suggesting that IRSp53 binding to PI(4,5)P₂ irrespective of the curved surface of I-BAR domain is sufficient to drive brush border assembly. The microvillar tip localization of IRSp53 is primarily governed by interaction with Eps8, a well-characterized interactor of IRSp53. Thus, in cells the interaction between IRSp53 and PI(4,5)P₂ only occurs at the tip of microvilli. Together, our data illustrate a previously unappreciated mechanism of brush border formation involving IRSp53 and PI(4,5)P₂.

P1689**Board Number: B205****Visualizing collective dynamics of endocytic proteins.**Y. Yang¹, M. Su¹, M. Wu¹;¹Mechanobiology Inst, Centre for BioImaging Sciences, Department of biological science, Natl Univ Singapore, Singapore, Singapore

Cdc42 and actin waves have been observed in multiple cellular contexts such as cell polarization, migration and divisions. Multiple models have proposed for the mechanisms of wave formation, with some involving vesicular trafficking and others not. In this work, we experimentally tested the

involvement of endocytosis in Cdc42 waves in stimulated mast cells 1 . Using total internal reflection fluorescence microscopy, we observed coordinated clathrin waves and Cdc42 waves. In addition, proteins involved in different modules of endocytic machinery(pit initiation, coat formation and scission) are sequentially recruited in the travelling waves in a manner similar to canonical clathrin-mediated endocytosis. Although clathrin may have intracellular localizations, assembly of these endocytic players, in particular, plasma membrane-specific adaptor proteins (AP2 and FCHO1) supports the plasma membrane association of the clathrin waves. Furthermore, the membrane identity of the travelling waves can be determined using optogenetic tool to deplete phosphatidylinositol 4,5-bisphosphate. Finally, perturbation of the endocytic machinery inhibits wave formation. Collectively our results indicates that endocytic waves not only accompanies but also upstream of actin and Cdc42 waves in stimulated mast cells.

Ref.:

1. Wu, M., Wu, X. & De Camilli, P. Calcium oscillations-coupled conversion of actin travelling waves to standing oscillations. Proc. Natl. Acad. Sci. U. S. A. 110, 1339–44 (2013).

P1690

Board Number: B206

Microtubule-dependent actin polymerization is necessary for growth cone protrusion in developing rat hippocampal neurons.

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Major cytoskeletal components, actin filaments and microtubules, play important roles in cells motility. Actin polymerization is the major mechanism to drive plasma membrane protrusion, adhesion and contraction in motile cells whereas microtubules are responsible for directional migration via poorly understood mechanisms. In neuronal growth cones, microtubules are believed to be capable of driving membrane protrusion in an actin-independent manner, because actin polymerization inhibitors enhance neurite elongation. We demonstrate contrary to this assumption that actin polymerization is necessary for neurite elongation in developing rat hippocampal neurons treated with varying concentrations of the actin polymerization inhibitors latrunculin B and cytochalasin D. Specifically, F-actin is invariably located at the tip of elongating neurites independent of the concentration of the inhibitors. We confirm that although neurites can grow longer than controls in the presence of low concentrations of the inhibitors, they cannot at higher concentrations, supporting the idea that neurite growth requires actin polymerization. Using platinum replica electron microscopy in combination with immunogold labeling, we report that branched actin filament networks physically associate with the tips of microtubules and occupy the foremost position at the leading edge of both drug-treated and untreated growth cones, whereas distal tips of microtubules lag behind. These data suggest that Arp2/3 complex-dependent actin polymerization, rather than growing microtubules, are responsible for neurite elongation in the presence of actin polymerization inhibitors. More importantly, our results suggest that actin

polymerization off microtubule tips can represent a mechanism by which microtubules guide directional cell migration.

P1691

Board Number: B207

Structure-function study of PICK1 and associated organelle motility.

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PICK1 (Protein Interacting with C-Kinase1) is a key regulator of receptor trafficking in neuronal cells and synaptic plasticity. PICK1 consists of an N-terminal PDZ domain, a central BAR domain, and a C-terminal acidic domain (CTail). The dimeric BAR domain mediates membrane binding and curvature inducing/sensing. The PDZ domain links PICK1 to the tails of receptors. Small angle x-ray scattering analysis yields a model that places the membrane-binding surfaces of the BAR and PDZ domains adjacent to each other, on the concave side of the banana-shaped PICK1 dimer. Full length PICK1 shows cytosolic distribution but it clusters on vesicles upon deletion of the CTail or PDZ domain. Activation is expected to expose the PDZ domain for binding to receptors at the plasma membrane. CTail containing PICK1 associated vesicles moves faster than the CTail deletion constructs. Thus, the CTail links PICK1-associated vesicles to motility factor, but in contrast to previous reports PICK1 neither binds nor activates Arp2/3 complex.

Kinesins 2

P1692

Board Number: B209

Development of single-molecule biophysical techniques for understanding the mechanochemical cycle of kinesin-1.

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Kinesin-1 is the most well studied member of the kinesin superfamily of molecular motors and is responsible for the trafficking of vesicles and organelles towards terminal branches of axons. This motor hydrolyzes ATP in a tightly coupled fashion in order to take directed 8 nm steps along a single

protofilament of a microtubule. However, the comprehensive mechanochemical cycle of kinesin-1 remains elusive, due largely to the technical limitations of fluorescence spectroscopy and microscopy approaches at the single-molecule level. Questions remain in identifying the rate limiting step in the hydrolysis cycle as well as the time spent in the one head bound state. To address these questions, we employ a fluorescence-free imaging technique, interferometric scattering microscopy (iSCAT), to track kinesin-1 at saturating ATP in a reconstituted system with greatly improved spatiotemporal resolution. With point spread function fitting, 8 nm and 16 nm steps are measured for C-terminus and N-terminus labeled motors, respectively. Using novel differences of means and Gaussian mixture model algorithms for the identification of step and sub-step regimes, we investigate the information present in both on-axis and off-axis positional data. Overall, these results point towards new insights into mechanochemical cycle of kinesin-1. The optical system and data analysis tools used present a new platform for investigating the stepping cycles of diverse molecular motors.

P1693

Board Number: B210

Transiently expressed Halo interacts with Kinesin-1 and Dynein to regulate lipid-droplet transport.

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In early *Drosophila* embryos, lipid droplets exhibit highly coordinated bidirectional transport, powered by Kinesin-1 and cytoplasmic Dynein. Motor activity on droplets is temporally regulated, resulting in global shifts of the entire droplet population as embryogenesis proceeds. Three phases can be distinguished (I, II, and III). The novel protein Halo is the key factor controlling this timing; its new expression mediates the switch to net plus-end transport in Phase II, by upregulating Kinesin-1 and downregulating Dynein activity on lipid droplets (Gross et al., 2003). During Phase III, Halo mRNA and protein levels drop sharply and net motion is minus-end directed. We find that in embryos mutant for the *lin-41/TRIM71* ortholog *Wech*, Halo mRNA and protein levels remain high and motion stays plus-end directed in Phase III; thus, abrogating Halo expression may be essential for the switch in net directionality. The objective is to understand how Halo acts molecularly. Previous work suggested three models: 1) Halo is a member of a small family, and one member, SNCF, has been proposed to be a co-factor of a transcriptional activator. 2) Halo is necessary for the Phase II-specific dephosphorylation of LSD-2, a lipid-droplet protein from the Perilipin family, essential for proper temporal regulation of transport. 3) Data from high-throughput screens suggest that several Halo family members physically interact with motors. Thus Halo might be a transcription factor, a phosphatase acting on LSD-2, or a motor cofactor. We find that Halo's intracellular distribution is consistent with all three models: Halo is present in nuclei as well as in the cytoplasm, and a small fraction copurifies with lipid droplets. Providing Halo (from injected *halo* mRNA) is sufficient to affect droplet motion even when transcription is pharmacologically inhibited; thus, Halo does not control transport by regulating transcription. Epistasis analysis revealed that Halo modulates transport even in the absence of LSD-2; thus Halo can act

independent of LSD-2. Using immunoprecipitation, we find that Halo and Kinesin-1 exist in common protein complexes in the embryo cytoplasm. Kinesin-1 and Halo indeed function in the same pathway as absence of either leads to failure of net plus-end transport during Phase II. Reducing Halo dosage delays this net plus-end transport, but – surprisingly – when levels of Halo and Kinesin-1 are reduced simultaneously, transport kinetics reverts back to wild type. We show that the critical parameter is the Kinesin-1/Halo ratio and propose that Halo is a limiting cofactor whose binding to Kinesin-1 increases motor processivity. Halo's inhibitory effect on Dynein may also be mediated by binding of Halo to the motor as Halo coimmunoprecipitates Dynein as well.

P1694

Board Number: B211

Kinesin-2 motor cooperation during cargo import, handover and transport unraveled at the single-molecule level in *C. elegans* cilia.

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Intraflagellar transport (IFT) is essential for building and maintaining cilia, the microtubule-based mechanosensory organelles of eukaryotic cells. In the chemosensory cilia of *Caenorhabditis elegans* two kinesin-2-family motors, kinesin-II and OSM-3, cooperate to drive IFT-trains that deliver ciliary cargo along the axoneme; consisting of doublet microtubules (the middle segment) that extend into singlet microtubules (the distal segment). Both kinesins are active in the middle segment, however, OSM-3 alone maintains and builds the distal segment. It is unclear how these two different kinesins collaborate and why their cooperation is beneficial to the IFT-system. Here, we study the dynamics of fluorescently labeled kinesins expressed at endogenous levels using ultrasensitive fluorescence microscopy to reach single-molecule sensitivity deep inside living *C. elegans*. Novel automated kymograph analysis tools and single-particle tracking analysis, provides unprecedented, quantitative insight into the cooperation between both kinesins. We reveal that the two kinesins have different roles, closely connected to their individual motility parameters. Kinesin-II, the slower and less processive motor, is mainly active at the ciliary base and the transition zone (a complex structure close to the base that is thought to function as a "ciliary gate"). Kinesin-II is responsible for loading IFT-trains and subsequent navigation through the protein dense transition zone. Once the IFT-trains have traversed this zone, kinesin-II gradually undocks, while the faster and more processive OSM-3 motor starts to dock on the IFT-trains propelling them towards the ciliary tip. This intricate, dynamic interplay between kinesin-II and OSM-3, ensures reliable entry, handover, and fast distribution of the IFT-trains. Our results demonstrate the power of *in vivo* single-molecule fluorescence microscopy to unravel motor cooperation and, more generally, motor-driven intracellular processes.

P1695**Board Number: B212****Conformational properties of a moving kinesin probed with the Tethered Cargo Motion assay.**E.V. Tarasovets¹, N.B. Gudimchuk², A.L. Drobyshev², B.D. Vitre³, V.V. Mustyatsa², D.W. Cleveland³, F.I. Ataullakhanov^{2,4}, E.L. Grishchuk¹;¹Physiology Department, University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA,²Center for Theoretical Problems of Physicochemical Pharmacology, Russian Academy of Sciences,Moscow, Russia, ³Ludwig Institute for Cancer Research and Department of Cellular and MolecularMedicine, University of California, San Diego, CA, ⁴Physics Department, Moscow State University,

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Members of the kinesin superfamily are characterized by a conserved motor domain but there is a significant variability in the length and predicted structure of their stalk domains. Centromere-associated protein E (CENP-E) is kinesin-7 with a 230 nm long discontinuous coiled-coil stalk, which based on electron microscopy imaging is predicted to be flexible (persistence length 26 nm). The biological role of this unusual stalk is not known but its highly extended length has been proposed to facilitate kinetochore capture of microtubules by searching a cytoplasmic volume. Interestingly, a mutant "Bonsai" version of CENP-E with a shortened stalk fails to support normal chromosome segregation during mitosis. To test whether CENP-E kinesin can provide a long reach from a cargo to microtubules we developed a modified Tethered Particle Motion (TPM) assay. Traditionally this method is used to analyze thermal motion of beads attached to a substrate via dsDNA molecules of a known length. However, microtubule-free CENP-E molecules are folded due to auto-inhibition, prohibiting analysis of this kinesin's conformation by TPM. Instead, we analyzed motions of the beads carried along the microtubules by the walking motors. To validate this approach we used a well-characterized kinesin-1, which was conjugated to the bead's surface via dsDNA 200 nm in length. We show that such assay, which we have called Tethered Cargo Motion (TCM) assay, provides a good measure of the tether length between the moving bead and microtubule, but the radial bead excursions can be underestimated due to several factors: non-specific motor absorption, multiple motor attachments and excessive density of dsDNA linkers. We then used the TCM assay to estimate the tether length formed by the walking CENP-E motor. We show that truncated CENP-E kinesin with no stalk behaves like a 2-5 nm tether, as expected. However, the full length CENP-E is not fully extended and behaves as a short tether with length.

P1696**Board Number: B213****Pavarotti/MKLP1 regulates microtubule sliding and neurite outgrowth in *Drosophila* neurons.**U. del Castillo¹, W. Lu¹, M. Winding¹, M. Lakonishok¹, V.I. Gelfand¹;¹Department of Cell and Molecular Biology, Northwestern University, Feinberg School of Medicine, Chicago, IL

Sliding of microtubules against each other, driven by kinesin-1, generates the mechanical force required for initial neurite extension in *Drosophila* neurons. This sliding is only observed in young neurons actively forming neurites and dramatically downregulated in older neurons. The downregulation of sliding is not caused by the global shut-down of kinesin-1, as the ability of kinesin-1 to transport membrane organelles is not diminished in mature neurons, suggesting that microtubule sliding is regulated by a dedicated mechanism. Here we identified a “mitotic” kinesin Pavarotti (Pav-KLP) as an inhibitor of microtubule sliding. Depletion of Pav-KLP from neurons strongly stimulated the microtubule sliding and neurite growth, while ectopic overexpression of Pav-KLP in the cytoplasm blocked both sliding and neurite extension. Furthermore, postmitotic depletion of Pav-KLP in *Drosophila* neurons in vivo reduced embryonic/larval viability, with only a few animals surviving to the third instar stage. In agreement with data observed in cultured neurons, surviving *elav>Pav* RNAi larvae showed overextended neurite outgrowth and mistargeting of motor neuron axons. Taken together, our results identify a new role for a “mitotic” kinesin as a negative regulator of neurite formation, and suggest an important parallel between microtubule-microtubule sliding in the mitotic spindle and sliding of interphase microtubules during formation of cellular processes.

P1697**Board Number: B214****Short stop mediates a regulatory actin-microtubule interaction to control cell shape.**M. Winding¹, V.I. Gelfand¹;¹Department of Cell and Molecular Biology, Northwestern University, Feinberg School of Medicine, Chicago, IL

The formation of processes by neurons is fundamental to neurodevelopment and the establishment of an interconnected nervous system. Neurons start as spherical cells and must break symmetry and extend processes called neurites. Our lab has recently demonstrated that microtubule-microtubule (MT) sliding by kinesin-1 is required for this dramatic change in cell shape. MT sliding occurs in many cell types besides neurons and yet these cells do not change shape or form cellular processes, suggesting regulatory mechanisms are present. In order to uncover this regulation, we utilized cultured *Drosophila* S2 cells as a model. Disruption of the actin filament (F-actin) network by the actin-depolymerizing toxin

Latrunculin B (LatB) greatly increased MT sliding by kinesin-1 in S2 cells. Strikingly, as MT sliding increased with increasing LatB concentration, cells began forming processes. This result suggests that F-actin inhibits MT sliding and also prevents sliding microtubules from forming processes. In order to determine how F-actin prevents process formation, we knocked down several actin-binding proteins with dsRNA. Depletion of the actin-microtubule crosslinking protein, short stop (shot), was sufficient to relieve the effects of F-actin on MT sliding. However, shot knockdown alone was not sufficient to relieve the inhibition of process formation by F-actin. Only combined shot depletion and loss of retrograde actin flow at the cell lamella by knockdown of an Arp2/3 subunit, Arpc4, allowed S2 cells to extend large >20µm processes without depolymerization of F-actin. These results suggest that the formation of processes is regulated by the combined action of shot crosslinking microtubules to actin and retrograde flow of the actin network preventing kinesin-powered microtubules from pushing on the membrane.

P1698

Board Number: B215

Depletion of Kinesin-12, a myosin IIB interacting protein, promotes rat cortical astrocytes migration.

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Kinesin-12 is a member of a group of microtubule-dependent motor proteins that are known to play key roles in cell division, contributing to the formation of the mitotic spindle, chromosome separation and cytokinesis. We have found that some of these motor proteins continue to be expressed in terminally post-mitotic neurons, where they contribute to important events such as migration of young neurons through the brain and the establishment of axons and dendritic arbors. Kinesin-12 is a mitotic motor that participates in all of these events during neuronal development, and appears to do so by contributing to microtubule movements and organization. However, the molecular mechanisms of kinesin-12 function remain to be revealed. In addition, little is known about the roles kinesin-12 may play in other cell types of the nervous system. In the present study, we have shown that depletion of kinesin-12 from cultured rat cortical astrocytes decreases cell proliferation but increases cell migration. We previously reported that kinesin-12 interacts either directly or indirectly with actin, which distinguishes it from kinesin-5, another motor with some overlap in functions both in mitosis and in neurons. Here we show that kinesin-12 directly interacts with myosin IIB, a non-muscle myosin II heavy chain, via their tail domains by CO-IP, GST pull-down and siRNA experiments. In addition, we used the fluorescence resonance energy transfer (FRET) method to show the interaction of kinesin-12 with myosin IIB protein. Finally, immunostain analyses revealed co-localization of kinesin-12 and myosin IIB in the lamellar region of the astrocytes. We posit that kinesin-12 and myosin IIB together can form a hetero-oligomer that uses force to integrate microtubules and actin filaments in certain regions of various cell types that comprise the developing nervous system.

P1699**Board Number: B216****Septin 9 interacts with KIF17 and regulates kinesin binding to cargo and microtubules.**X. Bai¹, E.T. Spiliotis¹;¹Biology, Drexel University, Philadelphia, PA

Septins are hetero-oligomeric GTP-binding proteins that assemble into non-polar filamentous structures, which associate with membranes and the microtubule and actin cytoskeleton. In mammalian cells, septins bind and bundle microtubules (MTs) and affect MT dynamics and post-translational modifications. Septins are also required for chromosome congression and biorientation during mitosis and vesicle transport in interface cells. Previous work has shown that SEPT7 interacts with the mitotic motor CENP-E (kinesin 7), but the function(s) of septins in kinesin-mediated cargo transport are unknown. Here, we have identified SEPT9 as a novel interactor of the kinesin-2 motor KIF17. We show that SEPT9 interacts strongly with the cargo-binding C-terminal tail of KIF17 and weakly with its motor domain. Pull down experiments show that SEPT9 interacts with the tail of KIF17 in E18 rat brain and MDCK cell lysates. In vitro binding assays show that the interaction is specific for SEPT9 as SEPT2/6/7 do not interact with the KIF17 tail. Conversely, SEPT9 does not interact with the C-terminal tail of the kinesin 13 motor MCAK. We show that SEPT9 interacts with the same domain of the C-terminal KIF17 tail that binds Mint1(mLin10), a scaffold protein that mediates the interaction of KIF17 with membrane cargo such as the NMDA (N-methyl-D-aspartate) receptor subunit 2B (GluN2B). Similar to the effects on the interaction of Mint1 with KIF17, mutations in the last 3 amino acids of the KIF17 tail decreased SEPT9 binding. Moreover, competitive in vitro binding assays showed that SEPT9 competes with Mint1 for KIF17 tail binding. Because the tail of KIF17 interacts weakly with MTs and the motor domain of KIF17, which is autoinhibited by its tail domain, we asked how these interactions are affected by SEPT9. Biochemical assays indicate that SEPT9 competes with the C-terminal tail for binding to MTs and conversely, enhances KIF17 motor-tail binding. Taken together, these data suggest that SEPT9 would inhibit both GluN2B-KIF17 cargo binding and KIF17 motor movement. Preliminary experiments in E18 rat hippocampal neurons suggest that SEPT9 may indeed interfere with transport of GluN2B and KIF17 to dendritic processes. Our results indicate that membrane- and microtubule-bound septins could regulate the motility of kinesins at the levels of cargo- and microtubule-binding, respectively.

P1700**Board Number: B217****Characterization of two novel kinesins that localize to distinct cytoskeletal structures in the human pathogen *Toxoplasma gondii*.**J.M. Leung¹, Y. He¹, F. Zhang², Y. Hwang³, E. Nagayasu⁴, J.M. Murray¹, K. Hu⁵;¹Department of Biology, Indiana University, Bloomington, IN, ²School of Medicine, University of Miami, Miami, FL, ³Nikon Instruments Inc., Melville, NY, ⁴Department of Infectious Diseases, University of Miyazaki, Miyazaki, Japan, ⁵Department of Biology, Indiana University Bloomington, Bloomington, IN

Toxoplasma gondii is a protozoan parasite that chronically infects ~20% of the global human population; it can cause life-threatening complications in immunocompromised individuals and is a leading cause of congenital neurological defects. This parasite is also a powerful model organism for studying aspects of cell biology shared with less experimentally tractable members of the phylum Apicomplexa, including *Plasmodium* spp., the causative agents of malaria. *T. gondii* has at least five tubulin-containing structures: the spindle pole, centrioles, conoid, intraconoid microtubules as well as cortical microtubules that spiral down two-thirds the length of the parasite. These play a number of important roles in the parasite including providing a scaffold for daughter cell construction during endodyogeny, structural stability and maintenance of parasite shape. However, little is known about the molecular motors that associate with these structures, and what role(s) they play during the lytic cycle of the parasite. Here we characterize two novel kinesins, TgKinesins 1 and 2, that were previously identified by proteomic analysis to be enriched in the conoid and cortical microtubule fractions, respectively. Both kinesins are conserved in several other apicomplexan parasites including *Plasmodium* spp., *Neospora caninum* and *Eimeria* spp. Fluorescently-tagged kinesins had unique subcellular distributions in knockin parasites. TgKinesin1 co-localized with a marker for the apical polar ring, which is thought to be a microtubule organizing center (MTOC) at the base of the conoid. In contrast, TgKinesin2 localized to the cortical microtubules, but was excluded from the most apical third of the microtubules as delineated by the markers TgCentrin2 and TgISP1. Fluorescence recovery after photobleaching (FRAP) experiments suggest that the diffusion rate and/or turnover of both TgKinesin1 and TgKinesin2 is slow in mature parasites. These data suggest that not only do TgKinesins 1 and 2 localize to distinct structures within the parasite, but that TgKinesin2 can only access a defined region of the cortical microtubules. Experiments to determine the functionality of these two intriguing microtubule-associated motors is currently underway.

P1701**Board Number: B218****Kinesin-2 mediated axonal transport facilitates synaptogenesis in *Drosophila* Central Nervous System.**S. Dey¹, R. Chakraborti¹, S. Shirolkar¹, A. Sadananda¹, A. Jain¹, M. Girotra¹, K. Ray¹;¹Department of Biological Sciences, Tata Institute of Fundamental Research, Mumbai, India

Axonal transport plays an important role in synapse assembly and maintenance in neurons. Synapse morphology and composition is severely affected due gross disruption of axonal transport in model organisms. In addition, abnormal axonal transport is predicted to cause synaptic dysfunction and progressive neurodegenerative disorders. In order to understand the correlation between selective axonal transport and synapse assembly, we studied the role of kinesin-2 based transport on synaptogenesis in developing central nervous system of *Drosophila* larvae. Here, we describe a fresh synaptogenesis event in the larval ventral ganglion during 78-84 hours After Egg Laying (AEL). Average number of synapses containing Dense Core Vesicles increases by three folds in this period. The step increase in synapse density is blocked in homozygous kinesin-2 mutants and ectopic expressions of appropriate transgenes in cholinergic neurons rescued the mutant phenotype. Kinesin-2 transports a variety of soluble and membrane associated cargoes in the axon which include choline acetyltransferase, acetylcholinesterase, rab4, etc. The synaptogenic phase is preceded by brief periods of kinesin-2 mediated ChAT and Rab4 (associated with presynaptic vesicles) transport in cholinergic neurons during 77-79 hours AEL. Altogether, these results provide a strong correlation between the Kinesin-2 dependent axonal transport and synapse assembly during development. In addition, it also suggests that specific loss of transport in cholinergic neurons could lead to synaptic atrophy and widespread neurodegeneration.

P1702**Board Number: B219****Aplip1 regulates Kinesin and Dynein during myonuclear movement in *Drosophila*.**A.L. Auld¹, E.S. Folker¹;¹Biology, Boston College, Chestnut Hill, MA

Myofibers, the cellular units of muscle, are a multinucleate cell type in which the many nuclei are positioned to maximize their internuclear distance. Although mispositioned nuclei correlate with muscle weakness, the nature of this correlation is poorly understood. Furthermore, how the destination for each nucleus is specified and the mechanisms by which each nucleus moves to its destination is not known. However, we have previously demonstrated that in vivo, translocating muscle nuclei are polarized with a distinct leading and lagging edge. We hypothesize that the polarized nature of translocating nuclei is evidence of the nucleus, or the molecules moving the nucleus, perceiving its eventual location and moving directly to this position. We have used genetic analysis to demonstrate that nuclear polarity requires the activity of Kinesin at the leading edge and Dynein at the trailing edge.

However, neither motor is polarized in its localization suggesting that only the activities of the two motors are spatially segregated. We have now screened a series of proteins known to regulate both Kinesin and Dynein to identify the mechanism of nuclear polarization, and eventually identified the mechanism by which nuclear position is specified. We identified JNK interacting protein 1 (Aplip1) as crucial to nuclear movement and nuclear polarization. Muscle specific RNAi-mediated depletion of Aplip1 resulted in mispositioned muscle nuclei. Additionally, Aplip1 interacts with both Kinesin and Dynein: Kinesin-Aplip1 double heterozygotes and Dynein-Aplip1 double heterozygotes both have defects in nuclear positioning. Time-lapse microscopy of nuclear movement in developing embryos further suggests that Aplip1 regulates the activity of each motor on the nucleus. This is illustrated by a reduction in nuclear movement velocity and an inhibition of the dynamic changes in the structure of both the leading and lagging edges of translocating nuclei. These data suggest that JNK signaling is essential for nuclei to polarize in the direction of their migration and achieve their maximal velocity. Further, these data suggest that JNK signaling may be a crucial indicator of the destination for individual nuclei.

P1703

Board Number: B220

Tubulation of Lysosomal Membranes Containing Niemann Pick Type C1 Requires StARD9 – the First Transmembrane Kinesin.

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Late Endosomes and Lysosomes (LE/Ls) are microtubule-dependent organelles that play an important role in cholesterol transport and acquire pathological accumulations of cholesterol in lysosomal storage diseases such as Niemann Pick Type C (NPC) disease. 95% of NPC cases are caused by mutations in NPC1, a 13-pass lysosomal transmembrane protein. LE/Ls containing NPC1 undergo alternating, bidirectional excursions along microtubules (MTs), and project dynamic membrane tubules that are also MT-dependent. Because the projection of dynamic membrane tubules is lost in cells expressing mutant but not wild-type NPC1, we used a proteomic survey to identify differences between these two membrane populations. StARD9 emerged as a novel membrane protein present in wild-type but absent in membranes containing I1061T mutant NPC1. This 4700 amino acid protein contains a conserved kinesin domain at the N-terminus, a dileucine, lysosomal targeting signal and a C-terminal StAR domain that overlaps with predicted transmembrane segments. StARD9 expression reveals MT-binding by N-terminal constructs and lysosomal accumulation by constructs also containing the StAR domain. Full-length StARD9 targets to lysosomes and incorporates into membrane tubules. ShRNA-driven depletion of StARD9 reduces centrifugal excursions of LE/Ls and projection of membrane tubules. These activities were rescued by shRNA-resistant wild-type but not a P-loop mutant StARD9 construct. Whereas co-expression studies reveal that wild-type NPC1 incorporates into lysosomes containing StARD9, mutant NPC1 fails to incorporate into StARD9-containing lysosomes. These findings identify StARD9 as the first

transmembrane kinesin and suggest that loss of StARD9 in mutant NPC1 membranes could explain defects in cholesterol transport.

P1704

Board Number: B221

Nesprins Anchor Kinesin-1 Motors to the Nucleus to Drive Nuclear Distribution in Developing Muscle Cells.

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During skeletal muscle development, nuclei move dynamically through the cell in a microtubule-dependent manner. The microtubule motor protein kinesin-1 drives nuclear dynamics in developing myotubes. Loss of kinesin-1 leads to improperly positioned nuclei in culture and in vivo. Two models have been proposed to explain how kinesin-1 functions to move nuclei in myotubes. In the cargo model, kinesin-1 acts directly from the surface of the nucleus, whereas in an alternative model, kinesin-1 moves nuclei indirectly by sliding anti-parallel microtubules. Here, we use two experimental strategies to test the hypothesis that an ensemble of Kif5B motors acts from the nuclear envelope to distribute nuclei throughout the length of syncytial myotubes. First, using an inducible dimerization system, we show that controlled recruitment of truncated, constitutively active kinesin-1 motors to the nuclear envelope is sufficient to prevent the nuclear aggregation resulting from depletion of endogenous kinesin-1. Second, we identify a conserved kinesin light chain (KLC) binding motif in the nuclear envelope protein nesprin-2 and show that recruitment of the motor complex to the nucleus via this LEWD motif is essential for nuclear distribution. Together, our findings demonstrate that the nucleus is a kinesin-1 cargo in myotubes and that nesprins function as nuclear cargo adaptors. The importance of achieving and maintaining proper nuclear position is not restricted to muscle fibers, suggesting that the nesprin-dependent recruitment of kinesin-1 to the nuclear envelope by binding of the conserved LEWD motif to kinesin light chain is a general mechanism for cell-type specific nuclear positioning during development. This work was supported by the National Institutes of Health [grant number PO1 GM087253 to E.L.F.H., T32 GM-07229 and T32 AR-053461 to M.H.W.]. Additional support was provided by the American Heart Association [#13PRE16090007 M.H.W.].

P1705**Board Number: B222****Mutual conformational changes of kinesin and GTP microtubule upon their binding.**M. Morikawa¹, H. Yajima¹, R. Nitta¹, S. Inoue¹, T. Ogura², C. Sato², N. Hirokawa³;¹Dept Cell Biol Anat, Univ Tokyo Grad Sch Med, Tokyo, Japan, ²Natl Inst Advanced Indus Sci Technol/AIST, Tsukuba Science City, Japan, ³Cell Biology and Anatomy, Univ Tokyo Grad Sch Med, Tokyo, Japan

The ATP-driven molecular motor kinesin moves along microtubules in an initial step coupled with ADP release. In neurons, kinesin-1/KIF5 preferentially binds to the GTP-state microtubules over GDP-state microtubules to selectively enter an axon among many processes; however, its molecular mechanism remains unresolved because the atomic structure of nucleotide-free KIF5 is unavailable. Here, the crystal structure of nucleotide-free KIF5 and the cryo-electron microscopic structure of nucleotide-free KIF5 complexed with the GTP-state microtubule are presented. The structures illustrate mutual conformational changes induced by interaction between the GTP-state microtubule and KIF5. KIF5 acquires the “rigor conformation”, where mobile switches I and II are stabilized through L7, L11 and the initial portion of the neck-linker, facilitating effective ADP release and the weak-to-strong transition of KIF5 microtubule affinity. Conformational changes to tubulin strengthen the longitudinal contacts of the GTP-state microtubule in a similar manner to GDP-taxol microtubules. These results and functional analyses provide the molecular mechanism of the preferential binding of KIF5 to GTP-state microtubules.

P1706**Board Number: B223****Direct measurement of the binding rate constant of kinesin to microtubules in living cells.**T. Kambara¹, Y. Okada¹;¹RIKEN Quantitative Biology Center (QBiC), Osaka, Japan

It has been established that conventional kinesin (kinesin-1, KIF5 in mammalian cells) selectively moves along a specific subset of microtubules in living cells. For example, KIF5 is specifically recruited to the microtubules in the axon initial segment in neurons, which would enable efficient transport into the axon. However, the mechanism of this selective binding is still controversial. Some groups have proposed that acetylation or other post translational modifications of tubulin serve as the cue for selective binding. We are proposing that conformational differences between the GTP-form and GDP-form of microtubules provide the cue. To test this idea, it would be important to examine whether kinesin binding to specific subsets of microtubules is enhanced, inhibited or both. Here, we measured the binding rate constant of kinesin to microtubules in living cells and in vitro using single molecule

fluorescence microscopy. To our surprise, the binding rate constant of KIF5 to the track-microtubule *in vivo* was nearly ten times higher than that *in vitro*, suggesting that mechanisms exist in the cell to recruit KIF5 specifically to some subset of microtubules by accelerating the binding reaction.

Dynein

P1707

Board Number: B224

Engineered tug-of-war between cytoplasmic dynein and kinesin *in vivo*.

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Microtubule (MT)-based transport is driven by minus-end directed cytoplasmic dynein and plus-end directed kinesins simultaneously bound to the surface of cargo organelles. Activities of opposing MT motors result in back-and-forth (“salutatory”) movement of cargo organelles along MT transport tracks. MT-based movement of organelles and cytoplasmic particles is tightly regulated in cells but the mechanisms of regulation remain largely unknown. The direction of MT-based transport might be controlled by a protein complex that determines which motor type is active at any given moment of time, or determined by the outcome of tug-of-war between MT motors dragging cargo organelles in opposite directions. However, evidence in support of each mechanism of regulation is based mostly on results of theoretical analyses or indirect experimental data. Here, we directly tested whether *in vivo* bidirectional movement of membrane organelles involves a tug-of-war between opposing MT motors by simultaneously activating cytoplasmic dynein and kinesin-1 on the same membrane organelles. For the activation of opposing MT motors, we took advantage of melanophores, which provide a dramatic example of regulation of MT-based transport. In these cells, pigment-containing melanosomes move along MTs to the cell center (pigment aggregation) or to the cell periphery (pigment dispersion) driven by cytoplasmic dynein and kinesin-2, respectively. Pigment aggregation signals boost dynein activity and inhibit activity of kinesin-2 as evidenced from a sudden and dramatic increase in the length of MT minus-end runs and a simultaneous decrease in the length of plus-end runs. To induce a tug-of-war between opposing MT motors, we attached to melanosomes large amounts of constitutively active kinesin-1 using the FKBP-rapalog-FRB heterodimerization system, and activated melanosome-bound dynein by applying pigment aggregation stimuli. We found that while melanosomes loaded with kinesin-1 failed to aggregate in the cell center, they continued bidirectional movement along the radial MT tracks. Measurement of movement statistics indicated that kinesin-1 shortened the length of dynein-dependent minus-end directed runs of melanosomes and also significantly (>20%) reduced their velocity. A decrease in transport velocity is consistent with application on dynein of an external load by the kinesin-1 motors. Activation of kinesin-2 by pigment dispersion stimuli never led to a decrease in velocity of minus-end runs of melanosomes. Our data show that forced simultaneous activation of

cytoplasmic dynein and kinesin-1 leads to tug-of-war between these motors and suggest that cellular mechanisms exist that regulate cargo runs in each direction thus preventing tug-of-war.

P1708

Board Number: B225

Regulation of dynein motility by the antenna structure of dynactin p150.

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Dynactin is an intricate complex and has been known as a dynein activator. Dynactin consists of 11 species of polypeptides, and among those p150 subunit constitutes a sidearm which extend from Arp1 rod. The coiled coil 1 region (CC1) of p150 is considered to bind directly to the N-terminal region of dynein intermediate chain (IC). Previously, we have shown that CC1 of p150 forms an antenna structure which extrude from the head within a sidearm. To elucidate the mechanism of dynein regulation by dynactin complex, we examined the effect of dynactin complex and the isolated CC1 on the dynein motility in vitro. Single molecules of cytoplasmic dynein diffusively moved on microtubules and exhibited slightly biased displacement to the minus end of microtubules as previously reported. Single molecules of dynactin complex showed diffusive movement on microtubules without unidirectional bias. When we mixed dynein and dynactin together, the fluorescent spot derived from dynein disappeared from microtubules. This was not caused by the competition of binding to microtubules between dynein and dynactin because both molecules are much fewer to occupy the binding sites on microtubules. Next, we prepared the recombinant CC1 and confirmed that the isolated CC1 directly bound to cytoplasmic dynein with a high affinity (K_d , ≈ 20 nM), but did not bind to microtubules. Single molecules of dynein associated with the CC1 released easily from microtubules and did not stay on microtubules for enough time to be detected, as is the case for the dynein-dynactin-complex. Intriguingly, when we added the CC1 to the gliding microtubules on the dynein coated surface, the moving microtubules gradually dissociated and finally disappeared from the surface. These observations suggest that the effect of CC1, the antenna structure of p150, on dynein motility is to reduce the affinity of dynein to microtubules, which appears to be inconsistent to the accepted idea that dynactin activates dynein motility by increasing its processivity.

P1709**Board Number: B226****Dynein, Dynactin and Lis1 are required for endosome maturation in *Drosophila* oocytes.**P. Sanghavi¹, G. Liu¹, N. Townes¹, G.B. Gonsalvez¹;¹Cellular Biology and Anatomy, Georgia Regents University, Augusta, GA

Several studies have demonstrated a requirement for Dynein in the transport of early and late endosomes. However, the extent to which Dynein contributes to endocytic uptake and maturation is poorly understood. Our initial experiments were focused on examining the role of p50/Dynamitin in oskar mRNA localization. Dynamitin is a component of the Dynactin complex, a core regulator of Dynein. Our findings indicate that Dynamitin is indeed required for efficient oskar mRNA localization in *Drosophila* oocytes. During the course of these studies, we unexpectedly discovered an additional role for Dynamitin in endocytic uptake and maturation. Large quantities of Yolk proteins are internalized into mid-stage oocytes via Clathrin-mediated endocytosis. Subsequently, maturation of these endocytic vesicles results in formation of condensed yolk granules. A defect in endocytosis is reflected by a reduction or loss of yolk granules. Our ultra-structural analysis indicated that oocytes depleted of Dynamitin contained significantly fewer yolk granules, but instead accumulated numerous endocytic intermediate structures. Particularly prominent were large vesicular structures that reflect an endocytic block downstream of Rab5. Similar endocytic defects were also observed upon depleting Dynein heavy chain or Lis1, another regulator of the Dynein motor. Consistent with a role for Dynein in endocytosis, we demonstrate that Dynein heavy chain is recruited to the oocyte cortex in an endocytosis-dependent manner. Our results suggest a model whereby endocytic activity recruits Dynein to the oocyte cortex. The motor in turn functions to ensure efficient endocytic uptake and maturation, ultimately resulting in the production of abundant yolk granules.

P1710**Board Number: B227****Molecular Mechanism for Dynactin Regulation of Processive and Diffusive Dynein Transport.**S.K. Tripathy¹, S.J. Weil², C. Chen³, S. Gross⁴, R. Vallee²;¹UC Irvine, Irvine, CA, ²Department of Pathology and Cell Biology, Columbia University, New York, NY,³biological sciences, Columbia university, New York, NY, ⁴Developmental and Cell Biology, UC Irvine, Irvine, CA

The dynactin complex is an important cytoplasmic dynein regulator implicated in both dynein cargo recruitment and motor regulation. Dynactin increases dynein run length along microtubules in *in vitro* assays, a function long thought to involve the N-terminal CAP-Gly microtubule-binding site within the major dynactin subunit p150^{Glued}. However, recent experiments from several labs argue against such a

role for this domain, and the mechanism for dynein regulation by p150^{Glued} remains unknown. We have produced and characterized well-behaved bacterially- and baculovirus-expressed subfragments of the N-terminal half of the major dynactin subunit p150^{Glued} and its neuronal splice variant p135, which lacks the CAP-Gly domain (Tripathy et al., ASCB 2013). We tested the effects of these constructs on dynein motor behavior by single molecule laser trap bead assays. We found that the largest p150^{Glued} and p135 fragments consisting of the N-terminal half of the full polypeptides each induce both diffusive and processive bead movement, as is the case for the complete dynactin complex. An internal fragment, CC1B, which is sufficient for dynein binding, was also sufficient to promote dynein processivity. The immediate upstream fragment, CC1A, increased dynein diffusion, whereas a complete CC1 fragment potentially inhibited dynein motility.

To address how these activities and that of the N-terminal globular domain of p150^{Glued} and p135 might be interrelated, we tested for interactions between domains. We identified a clear interaction between the individual CC1A and CC1B fragments using both size exclusion FPLC and pull-downs. In single molecule assays we also found clear CC1A dose-dependent neutralization of the ability of CC1B to stimulate dynein processivity. Together our data provide a comprehensive model for p150^{Glued}-mediated dynein regulation, involving CC1B as the prime locus for dynactin autoregulation by CC1A and, we speculate, by the N-terminal globular region of p150^{Glued}. Supp. by NIH GM102347 to RV and GM070676 to SG.

P1711

Board Number: B228

Dynactin functions as both a dynamic tether and brake during dynein-driven motility.

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Dynactin is a large multi-subunit complex that is an important cofactor for cytoplasmic dynein in cellular processes including organelle trafficking and mitosis. Dynactin increases the processivity of dynein and also targets dynein to specific cellular locations. Although the role of dynactin in dynein-mediated processes is well established, the underlying mechanisms involved are unknown. Here, we use single molecule approaches to investigate the contribution of the dynactin subunit p150Glued to dynein motor function. We first characterized the motility of purified GFP-tagged mammalian dynein isolated from a knock-in mouse brain tissue (Zhang et al., 2013). Consistent with previous reports (Mallik et al., 2005; Ross et al., 2006), single molecules of dynein-GFP switch stochastically from processive to diffusive states of motion. We analyzed this motion by a novel algorithm that we developed to parse trajectories into different states and found that dynein is processive 60% of the time. To examine the activation of dynein by dynactin, we investigated the formation and co-migration of a dynein-p150Glued co-complex using dual-color TIRF microscopy. We provide direct evidence that p150Glued is sufficient to recruit and tether dynein to the microtubule and that this recruitment is concentration-dependent; p150Glued

increases the on-rate and decreases the off-rate of dynein from microtubules. Single molecule imaging of motility in cell extracts demonstrates that the CAP-Gly domain of dynactin is essential for the decreased detachment rate of the dynein-dynactin complex from the microtubule and also acts as a brake to slow the dynein motor. Consistent with this important role, two neurodegenerative disease-causing mutations in the CAP-Gly domain abrogate these functions in our assays. Together, these observations support a model in which dynactin enhances the initial recruitment of dynein onto microtubules and promotes the sustained engagement of dynein with its cytoskeletal track. Thus, our work reveals new insights into the functioning of dynein and dynactin and how this process is perturbed in neurodegenerative disease.

P1712

Board Number: B229

Characterization of a dynactin p62 variant associated with increased risk of chronic *Pseudomonas* infection in cystic fibrosis.

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Dynactin is an essential component of the cytoplasmic dynein motor that contributes to interactions with dynein cargoes and other cellular components. The dynactin subunit p62 (DCTN4), along with Arp11, p27, and p25, comprise a subcomplex that is located distal to dynactin's dynein-binding site. We have shown that p62 is essential for dynactin complex integrity and targeting to specific cellular cargoes, but its full range of functions have not yet been defined. Exome sequencing has revealed that the presence of a single copy of the p62 variant (Y270C) in individuals with cystic fibrosis (CF) correlates with early onset of chronic *Pseudomonas aeruginosa* airway infection. We find that exogenously expressed p62-Y270C incorporates into the dynactin complex like p62-WT and does not affect dynactin integrity. p62 Y270, which is known to be phosphorylated *in vivo*, is a consensus site for kinases linked to cell adhesion and locomotion, so we investigated the impact of p62 Y270C on cell migration and focal adhesion dynamics. Cells expressing p62-Y270C migrate slower than those expressing p62-WT in a scratch wound assay. Centrosome reorientation in cells at the wound edge appears normal, but the morphology, location and number of actin stress fibers and focal adhesions (FAs) are altered. The level of the phosphorylated form of focal adhesion kinase (pFAK) at FAs is also significantly reduced, suggesting that the signaling at FAs may be perturbed. Live cell imaging showed that turnover of stress fibers (labeled with GFP-F-tractin) and FAs (labeled with RFP-zyxin) are also suppressed. Taken together, our results suggest that the p62-Y270C variant may reduce cell migration in the CF airway epithelium by altering stress fiber and FA dynamics, leading to decreased repair and increased susceptibility to infection. Further investigation of dynactin's contributions to cell migration is underway.

P1713**Board Number: B230****Dynein regulation by its microtubule track: Roles of tubulin carboxy-terminal tails *in vivo*.**C.E. Estrem¹, J.K. Moore¹;¹Cell and Development, University of Colorado School of Medicine, Aurora, CO

Dynein is a conserved microtubule motor with a multitude of cellular roles such as vesicle transport, mitotic spindle assembly, and spindle positioning. Despite these diverse roles, most eukaryotes express a single cytoplasmic dynein heavy chain; therefore, its activity must be highly regulated to provide specified force for different cellular functions. Here we identify a novel mode of regulation, in which dynein is regulated by tubulin carboxy-terminal tails (CTTs) that decorate its microtubule track. CTTs are negatively charged regions that differ across species and tubulin isotypes, and are major sites of posttranslational modification. We examine CTT mutants in budding yeast, where dynein generates force to move the mitotic spindle by sliding astral microtubules along the cell cortex. Using live cell imaging of fluorescently labeled microtubules, we demonstrate that truncation mutants that lack the β -tubulin CTT exhibit increased spindle movement. Microtubule sliding events in β -tubulin CTT mutants are more frequent and significantly longer-lived, producing greater displacement of the nucleus and mitotic spindle. These effects depend on dynein -- conditionally depleting dynein eliminates spindle movement in β -tubulin CTT mutants. Our results indicate that β -tubulin CTT negatively regulates dynein *in vivo*. We are investigating whether the β -tubulin CTT directly affects dynein's affinity for the microtubule or alters the activity of a different dynein regulator. This continued research offers new insight into how tubulins regulate microtubule binding proteins such as motor proteins, which in turn affects cellular processes.

P1714**Board Number: B231****The motility of axonemal dynein is regulated by the tubulin modifications.**J. Alper¹, F. Decker², B. Agana³, J. Howard¹;¹Molecular Biophysics and Biochemistry, Yale University, New Haven, CT, ²Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany, ³Chemistry, Missouri State University, Springfield, MO

Microtubule diversity, arising from utilization of different tubulin isotypes and post-translational modifications, regulates many cellular processes including cell division, centriole assembly, and neuronal differentiation. In cilia and flagella, microtubule diversity is important for axonemal assembly and motility. However, it is unknown whether microtubule diversity directly regulated the activity of the axonemal dyneins, the motors that drive the beating of the axoneme, or whether the regulation is indirect, perhaps through pathways upstream of the motors. To test whether microtubule diversity directly regulates the activity of axonemal dyneins, we asked if *in vitro* acetylation or deacetylation of

lysine 40 (K40), a major post-translational modification to tubulin, or proteolytic cleavage of the C-terminal tail (CTT) of tubulin, the location of detyrosination, polyglutamylation, and polyglycylation modifications as well as most of the genetic diversity, influences the activity of outer-arm dynein in motility assays using purified proteins. By quantifying motility, we found that K40 acetylation increases and CTTs decrease axonemal dynein motility. Further mathematical modeling suggests that these modifications regulate the power stroke speed of dynein, likely by altering the unbinding rate of the microtubule. These results show that axonemal dynein directly deciphers the tubulin code, which has important implications for eukaryotic cilia beat regulation.

P1715

Board Number: B232

Emergence of large-scale vortices of microtubules collectively driven by axonemal dyneins.

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Self-propelled particle models have been often used in trial that the motions often observed in biology (schools of fish, some flocks of birds, cell migrations during development and so on) are understood quantitatively. In addition to computer simulations of the models, substantial experimental systems have been demanded for the study and *in vitro* motility assays commonly used in protein-motors biophysics have provided some solutions. Using conventional *in vitro* motility assays, we reported collective behavior of microtubules driven by axonemal dynein molecules which are attached on glass surface [Sumino, Nagai, et al., *Nature* **483**, 448-452, 2012]. Under certain experimental conditions, the collective movement of microtubules generates nematic order, millimeter-scale meandering streams or millimeter-scale vortices. To explore the conditions causing such phase-shift, we have investigated collective behavior using different types of axonemal dynein subspecies and microtubules with various length. The motor proteins we used are the inner arm dynein subspecies c and g (dynein c and g) of *Chlamydomonas* flagella, both of which are single-headed dyneins composed of one heavy chain and two types of light chains, actin and p28 (for dynein c) or centrin (for dynein g). Dynein c and g are capable of moving microtubules on glass surface at velocities of ca. 12 and 6 $\mu\text{m}/\text{sec}$ in the presence of 1 mM Mg-ATP at 23 °C, respectively. Purified dynein molecules were adsorbed on a glass surface at densities higher than 1000 molecules μm^{-2} . Microtubules with length less than ca. 60 μm did not move in the absence of ATP but bound to dynein g-coated glass surface being slightly aligned by flow. Upon the addition of 1 mM Mg-ATP, microtubules started moving smoothly. Moving microtubules often collide each other and upon these collisions they are aligned through nematic interactions. Within a few minutes, microtubule-alignment gradually increases its size and finally forms streams meandering across a very large distance (longer than 400 μm). Under the experimental conditions with microtubules of less than ca. 60 μm , vortices with 200-500 μm diameter emerge from the stream tens of minutes after the ATP addition. On the other hand, microtubules with mean length of longer than 60 μm do not form

vortices. The features of vortices driven by dynein g, i.e. number, diameter and time necessary for their emergence, are different from those driven by dynein c. These results now suggest that the vortex formation is not dynein-c-specific but may reflect the mechanical properties of inner-arm dyneins. One parameters which can shift the phase is the length of microtubules but the mechanism how their length can modify the emergence still requires further investigation.

P1716

Board Number: B233

Regulation of Cytoplasmic dynein activity by GSK-3 β .

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Cytoplasmic dynein is a vitally important microtubule motor in animal cells, contributing to many aspects of cellular and intracellular motility. Genetic lesions that lead to altered dynein function have been associated with disease, but relatively little is known about signaling pathways regulating motor activity. Our studies now indicate that a prominent insulin response pathway culminating in inhibition of GSK-3 β (glycogen synthase kinase 3 β) contributes to dynein activation and increases minus end directed transport in both neuronal and non-neuronal cells. Moreover, GSK-3 β coprecipitates with dynein in mouse brain extract and phosphorylates dynein in vitro. Dynein phosphorylation by GSK-3 β reduces its interaction with Ndel1, a protein that contributes to dynein force generation. Because GSK-3 β inhibition occurs downstream of multiple receptor signaling pathways it has the potential to act as a switch, stimulating transport of dynein and local cargo away from peripheral membrane sites upon receptor activation.

P1717

Board Number: B234

Structure of human cytoplasmic dynein-2 primed for its powerstroke.

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Dyneins are a family of microtubule motor proteins belonging to the AAA+ (ATPases associated with diverse cellular activities) family. The cytoplasmic dynein-2 subfamily play a key role in intraflagella transport and in humans deficiencies are associated with a wide range of skeletal ciliopathies such as Jeune syndrome. Here we present the crystal structure of the human cytoplasmic dynein-2 motor bound to the ATP hydrolysis transition state inhibitor ADP.vanadate. This pre-powerstroke structure explains how the dynein motile element, called the linker, is bent and primed to generate force. It also reveals how the movements within the ring of AAA+ domains are coupled to the relative sliding of two α -helices in the dynein stalk that lead to release of dynein from microtubules.

P1718**Board Number: B235****Direct observation by cryo-EM of cytoplasmic dynein motors stepping along microtubules.**H. Imai¹, T. Shima², K. Sutoh³, M.L. Walker⁴, P.J. Knight¹, T. Kon^{5,6}, S.A. Burgess¹;¹School of Molecular and Cellular Biology and Astbury Centre for Structural Molecular Biology, Faculty of Biological Sciences, Univ Leeds, Leeds, United Kingdom, ²QBiC, RIKEN, Osaka, Japan, ³Waseda University, Tokyo, Japan, ⁴MLW Consulting, Cornwall, United Kingdom, ⁵Department of Frontier Bioscience, Hosei University, Tokyo, Japan, ⁶JST PRESTO, Saitama, Japan

Cytoplasmic dynein motor proteins perform critical roles in eukaryotic cells, transporting subcellular cargoes towards the minus ends of microtubule (MT) tracks and maintaining the positions of organelles. Tracking of fluorescently-tagged dimeric dynein suggested an uncoordinated stepping by the two heads, but there is also evidence of communication between them. We have now imaged directly by cryo-electron microscopy individual *Dictyostelium discoideum* dynein dimers flash-frozen while stepping along MT at near-physiological Mg-ATP concentration. About half of the molecules show diverse configurations of the two motor heads. The other half of the molecules has the two heads closely superposed with both their linker domains in post-powerstroke conformations. This unpredicted superposed configuration reveals more clearly the coiled-coil stalk domains and their terminal stalkheads that bind the dimer to the MT. The stalks are angled at $\sim 42 \pm 11^\circ$ to the MT, and are flexible mainly about the stalkhead-stalk junction, resulting in axial and radial variation of the heads. The superposed configuration is different from any configuration of the two other cytoskeletal motor families, kinesin and myosin, and thus direct motor-motor interactions may be functionally important in stepping by dynein.

P1719**Board Number: B236****The crystal structure of the dynein heavy chain dimerization domain.**A.G. Diamant¹, M.A. Schlager¹, H. Schmidt¹, M. Yu¹, A.P. Carter¹;¹MRC Laboratory of Molecular Biology, Cambridge, United Kingdom

Cytoplasmic dynein is a molecular motor responsible for the vast majority of retrograde traffic along microtubules. The largest subunit of the dynein complex is called the dynein heavy chain (DHC). The DHC includes a C-terminal motor domain, which converts ATP hydrolysis into mechanical force, and an N-terminal tail domain. An intermediate chain (DIC) and light intermediate chain (DLIC) bind directly to the DHC tail, while light chains (DLCs) bind to the DIC. This tail complex is important for both cargo binding as well as homodimerization of the DHC, which is necessary for processive movement. Previous studies suggest that the DLCs play an important role in homodimerization of the dynein complex, but it remains unclear whether the other subunits also contribute to the dimer interface.

Using *Saccharomyces cerevisiae* as a model, we co-expressed all four dynein subunits and purified functional dynein motors that were active in an *in vitro* motility assay. In this background, we found that truncating the DHC to include only the first ~1000 residues eliminates the motor domain, while preserving binding to the DIC, DLIC and DLC. Truncating another 50 residues off of the C-terminus led to a loss of all accessory subunits, suggesting this may disrupt a domain required for binding both the DIC and DLIC. Furthermore, a construct including only the first ~500 residues of the DHC dimerizes despite not being able to bind any of the other subunits. We solved the crystal structure of this smaller DHC fragment and found that the N-terminal ~200 residues of the DHC constitute an intricate dimerization domain. Not only is this the first crystal structure of any part of the DHC N-terminus, but it reveals a previously undocumented dimerization domain within the DHC itself. These studies shed new light on the biochemical organization of this important motor complex and contribute to a better understanding of its function.

P1720

Board Number: B237

Dynein light chain 1 locates at the stalk of the outer arm dynein.

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Cilia and flagella are highly conserved microtubule (MT)-based organelles in eukaryotes. The bending motion of cilia and flagella is driven by the motor protein called axonemal dyneins. The outer arm dynein (OAD) complex generates ~80% of propulsive force and thus OAD is an important component of cilia and flagella. OAD complex is composed of three heavy chains (α , β , γ HCs), two intermediate chains (ICs), and eleven light chains (LCs). Each of the three HCs comprises the tail and the head domains. Three HCs form a heterotrimer through their tail domains and thus the OAD complex appears as a three-headed structure. The head domain is composed of the linker, the AAA+ ring, the stalk, and the microtubule binding domain (MTBD). The stalk is a coiled-coil structure, which protrudes from the AAA+ ring, and the MTBD resides at the tip of stalk region. LC1 is a 22-kDa light chain of OAD and widely conserved among many species. While all the other smaller subunits (ICs and LCs) have been shown to associate with the tail domain, LC1 was found to associate with the head domain of the γ HC. LC1 was found to be essential for proper beating of cilia and flagella in many species. Mutation of human LC1 also leads to primary ciliary dyskinesia. Despite its importance *in vivo*, LC1's regulatory mechanism of OAD is not clearly understood because the structural basis for interaction of LC1 and γ HC has not been elucidated.

Here, by using single particle analysis of electron microscopy (EM), we have found that there is an additional density at the tip of the γ HC stalk region. By expressing His-tagged LC1 in *Tetrahymena* and *Chlamydomonas* and performing EM observation aided by Ni-NTA-nanogold labeling, we have shown

that this additional structure corresponds to LC1. This is in remarkable contrast to the previous assumption that LC1 locates at the AAA+ ring of the γ HC (Patel-King and King 2009). Pull-down assay using bacterially expressed fragments has shown that LC1 preferentially binds to the γ -stalk at a 1:1 molar ratio. Further biochemical mapping has revealed that the MTBD region of the γ HC is sufficient for the binding of LC1. These results raise the possibility that LC1 regulates OAD activity by changing γ HC's affinity to MTs.

P1721

Board Number: B238

Characterizing the Role of Cytoplasmic Dynein in HTLV-1 Intracellular Trafficking.

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Human T-Cell Leukemia Virus type 1 (HTLV-1) is a human retrovirus that is associated with a variety of human diseases, which include adult T-Cell leukemia/lymphoma (ATLL) and HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP). A hallmark for retroviral infection requires the integration of the viral genome into the host cell genome. One problem the virus faces is trafficking through the cytoplasm in order to reach the nucleus. Some viruses have overcome this obstacle by hijacking molecular motors that move through the cytoplasm on microtubules (MT) in a retrograde fashion. The motor protein, termed cytoplasmic dynein, mediates retrograde movement of intracellular cargo along microtubules. In previous experiments, our laboratory has demonstrated that HTLV-1 particles co-localized with cytoplasmic dynein and dynactin within HEK293T cells. Also immunoprecipitation studies revealed that HTLV-1 directly or indirectly interacts with cytoplasmic dynein in-vitro. Thus, we have hypothesized that HTLV-1 utilizes cytoplasmic dynein to travel to the nucleus in order to insert its DNA into the host genome. To investigate the role of cytoplasmic dynein in HTLV-1 trafficking, we have optimized conditions to perform experiments using short interfering RNAs (siRNA). By inhibiting the expression of this protein, we can better understand the role cytoplasmic dynein has in retrovirus movement. Through these studies, we expect to further elucidate the molecular details of HTLV-1: host cell interactions. Furthermore, these studies should reveal insights to potential therapeutic targets.

P1722**Board Number: B239****A Ras-Like Domain in the Light Intermediate Chain Bridges the Dynein Motor to a Cargo-Binding Region.**C.M. Schroeder^{1,2}, J.M. Ostrem^{1,2}, N.T. Hertz^{1,2}, R.D. Vale^{2,3};¹University of California, San Francisco, San Francisco, CA, ²The Howard Hughes Medical Institute, San Francisco, CA, ³Department of Cell and Molecular Pharmacology, University of California, San Francisco, San Francisco, CA

Cytoplasmic dynein, a microtubule-based motor protein, transports many intracellular cargos by means of its light intermediate chain (LIC). However, it is not understood how the LIC carries out intracellular transport, and structural information about the LIC is lacking. We have determined the crystal structure of the conserved LIC domain, which binds the motor heavy chain, from a thermophilic fungus. We show that the LIC has a Ras-like fold with insertions that distinguish it from Ras and other previously described G proteins. Despite having a G protein fold, the fungal LIC has lost its ability to bind nucleotide, while the human LIC1 binds GDP preferentially over GTP. We show that the LIC G domain binds the dynein heavy chain using a conserved patch of aromatic residues, whereas the less conserved C-terminal domain binds several Rab effectors involved in membrane transport. These studies provide the first structural information and insight into the evolutionary origin of the LIC as well as revealing how this critical subunit connects the dynein motor to cargo.

P1723**Board Number: B240****Dual Role for Cdk1 in Triggering Early and Late G2 Dynein Recruitment to the Nuclear Envelope.**A. Baffet¹, D.J. Hu¹, R. Vallee¹;¹Department of Pathology and Cell Biology, Columbia University, New York, NY

Cytoplasmic dynein is recruited to the nuclear envelope (NE) during G2 via two distinct nuclear pore-anchored mechanisms involving, respectively, RanBP2-BicD2-dynein/dynactin/Lis1 and Nup133-Cenp-F-NudE/L-dynein (Splinter et al, PLoS Biol., 2010; Bolhy et al., JCB, 2011). These pathways also function in neuronal progenitor cells, where they control early vs. late G2 apical nuclear migration (Hu et al., Cell, 2013). How they are activated specifically during G2 is unknown. This question has particular relevance in neuronal progenitors, in which nuclei must reverse their direction of transport from kinesin-dependent basal movement during G1 to dynein-dependent apical movement to the ventricular surface of the developing brain during G2. Failure to reach the ventricle blocks mitotic entry and neurogenesis. To address this important question we have undertaken experiments to test the role of cell cycle-dependent protein kinases in NE dynein recruitment and nuclear migration. We previously found that small molecule Cdk1 inhibitors block the recruitment of dynein, as well as dynactin and Lis1 to the HeLa

cell NE and inhibit apical nuclear migration in the developing rat brain (Baffet et al., ASCB 2013 abstract). We have now further characterized the underlying molecular mechanism for Cdk1 action. We show that Cdk1 inhibition displaces the most upstream factors in each pathway, BicD2 and Cenp-F, whereas Cdk1 stimulation results in more intense and even earlier BicD2 and Cenp-F NE decoration. We identify novel Cdk1 phosphorylation sites in the BicD2-binding region of RanBP2, phosphorylation of which strongly enhances binding to BicD2-CT in vitro. Constitutive targeting of BicD2-NT to the NE of Cdk1-inhibited cells restores dynein, dynactin and Lis1 recruitment to the HeLa cell NE and rescues apical nuclear migration in neuronal progenitor cells. These results reveal that Cdk1 phosphorylation of factors downstream of BicD2 (dynein, dynactin, Lis1) is not involved in their targeting to the NE. Surprisingly, Cdk1 inhibition affects the late dynein NE recruitment pathway by preventing Cenp-F from exiting the nucleus. In conclusion, Cdk1 independently controls both the early and late NE dynein recruitment pathways. Cdk1 phosphorylates RanBP2 to activate BicD2 recruitment to the NE, which appears to serve as the trigger for apical nuclear migration in neuronal progenitor cells. Cdk1 also activates the late NE dynein recruitment pathway by a novel mechanism, controlling the exit of Cenp-F from its default intranuclear localization. Supported by HD40182 to RV and an AHA PD Fellowship to AB.

P1724

Board Number: B241

Differential Roles for NudE1 and NudEL1 In Rat Brain Development.

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During development of the mammalian neocortex, radial glial progenitor (RGP) cells divide while undergoing cell cycle dependent interkinetic nuclear migration (INM). We have found apical nuclear migration to be dependent on cytoplasmic dynein, recruited to the nuclear envelope (NE) by sequential G2-specific mechanisms (Hu et al. *Cell*. 2013, 154:1300-13), which also control G2-dependent dynein recruitment to the NE in cultured non-neuronal cells. The specific roles of two of these component proteins – NudE and NudEL – in the NE dynein recruitment process, however, remain unexplored. NudE and NudEL are closely related throughout their length (55% identity; 70% similarity), and each interacts with cytoplasmic dynein and Lis1. However, knockout mice exhibit different brain developmental phenotypes (Feng & Walsh. *Neuron*. 2004, 44:279-93; Sasaki et al. *Mol Cell Biol*. 2005, 25:7812-27), consistent either with different functional roles or distinct brain developmental expression patterns. Using in utero electroporation of Nde1 and Ndel1 shRNAs in E16 rat brains, followed by fixed or live imaging in brain slice culture, we tested the effects of NudE and NudEL RNAi on neurogenesis and migration. NudE RNAi resulted in severe inhibition of apical INM, and strongly reduced the number of dividing cells at the ventricular surface. Ectopic divisions away from the ventricular surface were undetectable. In contrast, depletion of NudEL had no effect on INM or the frequency of dividing cells at the ventricular surface. RNAi for each gene led to an accumulation of multipolar cells in the intermediate zone, and severe inhibition of further neuronal migration of postmitotic neurons in the cortical plate. To test for inherent differences in NudE and NudEL function, we have begun series of

complementation experiments. We find that the post-mitotic neuronal migration defect seen in NudEL RNAi is largely rescued by NudE overexpression. Thus, NudE and NudEL may exhibit significant functional redundancy in the brain. Of further importance, the specific inhibition caused by NudE RNAi in INM, and the failure of NudE knockdown cells to reach the ventricle to divide, may play a substantial and previously unappreciated role in the clinical microcephaly reported in human patients with mutations in NDE1 (Alkuraya et al., *Am J Hum Gen.* 2011, 88:536-547). Supp. by NIH HD40182.

P1725

Board Number: B242

NuMA links the mitotic spindle with plasma membrane lipids.

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Correct positioning of the mitotic spindle during metaphase and spindle elongation during anaphase are critical for error-free mitosis. In human cells, the evolutionary conserved ternary complex components NuMA/LGN/G α i1-3 anchor dynein at the cell cortex, thus ensuring metaphase spindle positioning. Whether the ternary complex also participates in cortical localization of dynein during anaphase was previously not known. Importantly, mechanisms by which mitotic spindle behaviour is coupled with mitotic progression remained elusive. We discovered that cortical levels of dynein markedly increase as cells transit from metaphase to anaphase, and this in a NuMA- and CDK1-dependent manner. We showed also that this increase of cortical dynein is crucial for proper spindle elongation during anaphase. Notably, we uncovered that LGN/G α i1-3 are dispensable for NuMA-dependent cortical dynein enrichment during anaphase. In addition, we established that NuMA is excluded from the cortical equatorial region by the centralspindlin complex components CYK4 and MKLP1. Interestingly, we discovered that NuMA directly interacts with membrane phosphoinositides in vitro and in vivo, and that cortical localization of NuMA during anaphase is dependent on these lipids. In summary, our study uncovered a novel mechanism by which NuMA links cell cycle progression with proper spindle behavior by directly associating with membrane lipids to ensure faithful execution of mitosis.

P1726

Board Number: B243

The dynein cortical anchor Num1 activates dynein motility by relieving Pac1/LIS1-mediated inhibition.

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Cortically anchored dynein motors orient the mitotic spindle through interactions with spindle pole anchored astral microtubules. In budding yeast, dynein is offloaded to cortical Num1 receptor sites from

the plus ends of dynamic microtubules. Rather than walking toward minus ends, dynein remains associated with microtubule plus ends due to its association with Pac1 (LIS1 homolog), a potent inhibitor of dynein motility. The mechanism by which dynein is switched from “off” at the plus ends to “on” at the cell cortex remains unknown. Here we show that overexpression of the dynein-interacting coiled-coil domain of Num1 (Num1CC) – which is insufficient for cortical anchoring – depletes dynein-dynactin-Pac1 complexes from microtubule plus ends, and reduces the extent of colocalization of dynein and Pac1. Num1CC-mediated depletion of dynein from plus ends requires an interaction between Num1 and dynein, and also requires the dynein microtubule-binding domain. Live cell imaging reveals minus end-directed motility of dynein molecules along astral microtubules upon Num1CC overexpression – an event that is never observed in wild-type cells. An enhanced Pac1-affinity mutant of dynein, or overexpression of Pac1 partly restores Num1CC-mediated depletion of dynein from plus ends, suggesting that Num1CC activates dynein-dynactin motility by inducing the release of Pac1. Our findings indicate that dynein activity can be directly switched “on” by Num1, which, upon binding to dynein-dynactin complexes, may induce the removal of the Pac1/LIS1 inhibitor.

P1727

Board Number: B244

Budding Yeast She1 Restricts Loading of Ipl1/Aurora B onto the Spindle to Coordinate Spindle Integrity with Spindle Positioning.

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In budding yeast, the mitotic spindle protein She1 is a key regulator of dynein pathway activity that functions to dampen productive microtubule-cortex interactions in the mother cell in order to achieve polarized spindle movements toward the bud. While the role for She1 is well established in the cytoplasm, its role in the nucleus is poorly understood. Here, we show that She1 is required for proper timing of Ipl1/Aurora B recruitment onto the mitotic spindle to ensure spindle integrity for dynein-dependent pulling from the cell cortex. Ipl1/Aurora B localizes in the nucleus with a diffusive pattern during metaphase and loads onto the mitotic spindle immediately prior to anaphase onset. Loss of She1 leads to premature loading and enhancement of Ipl1 on the spindle, whereas overexpression of She1 delays loading. In *she1Δ* metaphase spindles, enhanced Ipl1 resulted in a decreased level of microtubule-stabilizing +TIP protein Bim1 and an increased rate of anaphase spindle elongation due to cortical dynein activity. Additionally, *she1Δ bim1Δ* double mutant exhibited a synthetic broken spindle phenotype. The N-terminal conserved region of She1 is sufficient for restricting Ipl1, whereas the C-terminal domain is required for dampening cortical dynein. Our results establish a new role for She1 in ensuring spindle integrity through restricting Ipl1 in the nucleus, while promoting spindle positioning through cortical dynein.

P1728**Board Number: B245****Development and characterization of a cytoplasmic dynein mutant mouse carrying a human Charcot Marie Tooth mutation.**S. Nandini¹, T. Sabblah¹, N. Patwardhan¹, A. Ledray¹, V. Nascimento¹, J. Pasos¹, R. Love¹, L. Ehler¹, S.J. King¹;¹Burnett Sch. Biomed. Sci., Univ Central Florida, Orlando, FL

Charcot Marie Tooth disease (CMT) is the most common inheritable peripheral neuropathy in the United States. Individuals with CMT experience a range of symptoms, ranging from mild loss of motor coordination and some muscle weakness to debilitating pain and muscle weakness, often leading to the need for therapeutic intervention. Dynein is an essential molecular motor that functions to transport cargos in all cells. Multiple human dynein mutations have recently been shown to cause a variety of neurological disorders, including CMT, suggesting that dynein transport along nerve axons may be more sensitive than in other cells to genetic and physical dysfunction. To test this hypothesis we developed and are characterizing a novel mutant mouse model that carries a human dynein mutation found in a hereditary form of CMT. We found that heterozygous dynein^{CMT}/+ mice have motor skills behavioral phenotypes that are consistent with a loss of muscle strength and/or muscle coordination. The phenotypes we observed appear to be distinct and less severe than the motor skills phenotypes seen in Cra, Loa, and Swl mutant dynein mice, which do not have corresponding mutations in humans. Most interesting, homozygous dynein^{CMT}/dynein^{CMT} mice have survived up to 9 months and counting, whereas Cra/Cra, and Loa/Loa mice die within 24 hours of birth. We have determined that homozygous dynein^{CMT}/dynein^{CMT} mice have more severe motor skills behavioral phenotypes. With this mouse model in hand, it is now possible to study the effect of a known human disease causing DHC mutation on development, whole animal neurodegeneration, and axonal transport at the cellular level. In vitro analyses of pure mutant protein can now be performed without the confounding background of wildtype DHC dimers or mixed DHC/+ dimers. By correlating the behavioral phenotypes with the molecular characterization of the mutant dynein and cellular transport assays, we hope to generate a more comprehensive understanding of the key role dynein plays in neurological disease, ultimately leading to better human therapeutics including potential drug intervention strategies.

P1729**Board Number: B246****Role of NDE1 in T cell effector functions.**S. Nath¹, S.F. Lee², J. Artz³, S. Wong¹, M. Poenie¹;¹Department of Molecular Biosciences, The University of Texas at Austin, Austin, TX, ²Neogenomics Laboratories, Fort Myers, FL, ³Graduate School of Biomedical Sciences, University of North Texas Health Science Center, Fort Worth, TX

Helper and cytotoxic T cells exert their effector functions mainly through focused secretion. This process is thought to be accomplished by clustering of secretory vesicles at the centrosome or microtubule organizing center (MTOC) and translocation of MTOC up to the site of contact (the immunological synapse or IS). Our studies and those of others have implicated dynein in the process of MTOC translocation. Here we have examined the role of the dynein-binding protein NDE1 in the process of MTOC translocation. We recently found that NDE1 is recruited to the immunological synapse when Jurkat T cells engage superantigen-coated Raji cells or when cells from an NK cell line (NK92) engage Daudi cells. In both the cases both NDE1 and dynein form a ring-like structure corresponding to the pSMAC of the immunological synapse. Immunoprecipitation of either dynein or NDE1 show that both proteins form a complex that includes Lis1. When expression of NDE1 was reduced using siRNA, MTOC polarization process was reduced nearly to levels of a negative control. Moreover, when NDE1 expression was reduced by siRNA, dynein failed to localize at the synapse. Depletion of NDE1 did not interfere with other aspects of T cell activation such as accumulation of actin or phosphorylated LAT at the synapse or calcium signaling in response to stimulation with anti-TcR mAb. Localization of NDE1 was also examined in live cells using GFP-NDE1 chimeras. Interestingly, studies of GFP-NDE1 (GFP at the N-terminus) showed that the expressed construct failed to accumulate at the synapse, that it completely inhibited MTOC translocation, and that it displaced dynein from the synapse. On the other hand, expressed NDE1-EGFP (C-terminal EGFP) did accumulate at the synapse and did not interfere with either MTOC translocation or accumulation of dynein at the synapse. Dynein forms mutually exclusive complexes either with dynactin or with NDE1 and Lis1. These results combined with previous studies of Lis1 and dynein trapping using molecular traps show that the dynein-Lis1-NDE1 axis is required for MTOC translocation.

P1730**Board Number: B247****Characterization of Novel Splicing of Dynein's Microtubule-Binding Domain.**S. Bernard¹, J. Wildonger²;¹Integrated Program in Biochemistry, University of Wisconsin-Madison, Madison, WI, ²Biochemistry, University of Wisconsin-Madison, Madison, WI

The distribution of mRNAs, proteins and organelles within cells depends on the activity of molecular motors. While the kinesin family of motor proteins includes many members that transport cargo to the

plus-ends of microtubules, dynein is the major cytoskeletal motor responsible for minus-end-directed transport along microtubules. We have identified novel alternative splicing of the microtubule-binding domain in the *Drosophila* dynein heavy chain gene (Dhc64C) encoding the motor subunit of dynein. This alternative splicing affects helices within the microtubule-binding domain that are predicted to directly interact with microtubules. These isoforms are expressed throughout *Drosophila* development and in the adult. To determine the biological function of alternatively spliced microtubule-binding domains, we are utilizing the CRISPR-Cas9 system to generate animals that selectively express either isoform. We are also analyzing the microtubule-binding domain of the heavy chain gene in other organisms to determine how this alternative splicing evolved. These studies of novel microtubule-binding domain splicing will serve to define the effect of microtubule-binding domain residues upon dynein function.

P1731

Board Number: B248

Initial Characterization of a Probe to Measure Microtubule Sliding Forces in Living Cells.

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Microtubules play critical roles during many cellular processes, often functioning directly in the transport and movement of various intracellular components. Microtubules can affect these movements actively by using energy stored in their lattice to push or pull, or passively by acting as a scaffold upon which motor proteins and their associated cargoes can travel. In either capacity, microtubules must bear loads imparted by forces. Thus, full understanding of microtubule function within the cell will require measuring and characterizing these forces. Toward this end, we have developed a genetically-encoded probe designed to measure the forces that slide microtubules relative to one another in living cells. This probe consists of a FRET-based tension sensor flanked on either side by microtubule binding domains derived from tau. Truncated versions of this probe decorate spindle microtubules homogeneously, in contrast to similar probes derived from the microtubule binding domain of ensconsin. Using *in vitro* microtubule gliding assays, we demonstrate that the full-length tension sensor can crosslink microtubules and that it is indeed sensitive to the forces that are generated between them. Future work will involve calibration of the tension sensor, development of single molecule imaging strategies, and construction of a spatial map of microtubule sliding forces in the mitotic spindle.

P1732**Board Number: B249****Role of Dynactin in HTLV-1 Infection.**T.M. Gibson¹, R. Sperl¹;¹Biology, University of Texas of the Permian Basin, Odessa, TX

The cytoskeleton is responsible for overall cell shape and structure, as well as movement and organization of cellular organelles. Motor proteins, which associate with components of the cytoskeleton, facilitate many different types of intracellular movement. This includes organization of organelles and movement of chromosomes during cell division. Cytoplasmic dynein is a motor protein complex that mediates some of the retrograde movement in the eukaryotic cell. Dynein is known to interact with an activator protein called dynactin, which has been proposed to be critical for most of cytoplasmic dynein's activity. Evidence has shown that viruses, such as adenovirus and retroviruses, utilize the cytoskeleton for intracellular movement. One retrovirus termed Human T-cell Leukemia Virus-1 (HTLV-1) exhibits microtubule-dependent movement. HTLV-1 is a retrovirus that can cause a rare blood cancer called Adult T-cell Leukemia (ADL). HTLV-1 movement toward the nucleus during the infection process is poorly understood. Our laboratory has evidence that HTLV-1 binds to the dynein motor protein complex. Therefore, we hypothesize that dynactin is required for HTLV-1 intracellular movement. To investigate the role of dynactin in HTLV-1 movement during infection, siRNA will be used to knockdown dynactin expression in HEK 293 T-cells. The dynactin siRNA is not tagged, therefore a GFP-tagged GAPDH siRNA knockdown will be performed simultaneously to determine transfection efficiency of the dynactin knockdown. Next, the cells be infected with GFP-tagged HTLV-1, and infection and intracellular movement will be monitored using fluorescent microscopy. It can be predicted that HTLV-1 will not be able to properly attach to the dynein motor protein complex, resulting in little to no retrograde movement based on the fact that dynactin is required for most of dynein's activity. Western blot analyses of knockdown cell lysis have revealed a decreased GAPDH expression. Additionally, quantitative analysis of these Western blots has shown a 90 – 95% reduction in GAPDH compared to control samples. If a dynactin knockdown results in limited intracellular movement of HTLV-1, these results will reveal new information about the mechanism used by viruses to maneuver throughout the host cell. Additionally, antivirals and therapeutics could be developed to stop this interaction, thus halting the viral cycle.

P1733**Board Number: B250****Veza, a novel factor required for dynein-mediated early endosome transport.**X. Yao¹, H.N. Arst², X. Wang³, X. Xiang¹;¹Department of Biochemistry and Molecular Biology, USUHS - F. Edward Hébert School of Medicine, Bethesda, MD, ²Microbiology Section, Department of Medicine, Imperial College London, London, UK,³Department of Plant Genetics and Breeding, College of Agronomy and Biotechnology, China Agricultural University, Beijing, China

Cytoplasmic dynein transports organelles/vesicles and proteins/mRNA along microtubules. However, how dynein function is regulated *in vivo* remains unclear. We have been using *Aspergillus nidulans* as a model organism to dissect the regulation of dynein-mediated transport of early endosomes. Using a classical genetic approach, we have identified genes that are required for dynein-mediated early endosome distribution, and we recently reported the identification of *A. nidulans* HookA and FhipA proteins that link dynein-dynactin to early endosomes (Zhang et al., 2014; Yao et al., 2014). From the same genetic screen that allowed us to identify HookA and FhipA, we have recently identified Veza (vezatin-like protein in *A. nidulans*), another novel factor critical for dynein-mediated early endosome transport. The identification of the *veza* gene was achieved by using a combination of classical genetic and whole genome sequencing approaches. The Veza protein contains a vezatin domain that shows weak similarity to the mouse vezatin protein and the budding yeast protein Inp2p. Vezatin in mammalian cells binds myosin VIIA to bring it close to the cadherin-catenins complex (Küssel-Andermann et al., 2000), while the yeast Inp2p is a peroxisomal receptor for the myosin V protein Myo2p (Fagarasanu et al., 2006). Our current data show that, unlike HookA or FhipA that associate with early endosomes, Veza does not co-localize with early endosomes. In addition, Veza is not required for HookA-early endosome interaction, which is in contrast to the function of FhipA. We are in a process of further studying the mechanism of Veza action in dynein-mediated early endosome transport.

P1734**Board Number: B251****Functional analyses on the p25 and p27 components of dynactin in *Aspergillus nidulans*.**R. Qiu¹, J. Zhang¹, X. Xiang¹;¹Department of Biochemistry and Molecular Biology, USUHS - F. Edward Hébert School of Medicine, Bethesda, MD

Within the dynein-accessory complex dynactin, the two left-handed beta-helical proteins p25 and p27 locate at the pointed-end of the Arp1 mini-filament and are required only for a subset of dynein functions (Eckley et al., 1999; Lee et al., 2001; Schroer 2004; Parisi *et al.*, 2004; Zhang et al., 2011; Yeh et al., 2012; 2013). In *Aspergillus nidulans*, dynein-mediated early endosome transport requires p25, which is necessary for dynactin-dynein to interact with the early endosome adapter protein, HookA (A.

nidulans Hook) (Zhang et al., 2011; 2014). In the vertebrate dynactin complex, p25 and p27 form a heterodimer (Eckley et al., 1999), and RNAi of p25 makes p27 unstable and vice versa (Yeh et al., 2012). Thus, whether it is p25 or p27 that is more directly involved in early endosome transport and HookA-dynein-dynactin interaction would need to be addressed. Here we found that in *A. nidulans*, loss of p27 does not apparently affect p25-dynactin-dynein interaction or p25 protein level, but loss of p25 abolishes p27-dynactin-dynein interaction and reduces the level of p27. However, p25 function in early endosome transport is not mediated by p27 because loss of p27, unlike loss of p25, does not significantly affect HookA-dynein-dynactin interaction and dynein-mediated early endosome movement. We performed homology-based modeling of p25 structure and predicted that *A. nidulans* p25 contains, besides the core structure of the left-handed beta-helix core, two loop regions and a C-terminal alpha helix. We also identified several amino acid clusters whose side chains face outside of the core structure. Our functional analysis revealed three peripheral regions of p25 that contribute to dynein-mediated early endosome transport. These regions include Loop-1, the C-terminal region and the K36-Y83-H113 cluster. Importantly, these amino acids only play a minor role in p25-dynactin-dynein association but play a significant role in HookA-dynein-dynactin interaction. These data support the notion that within the dynactin complex, p25 is the protein specifically required for mediating HookA-dynein-dynactin interaction.

P1735

Board Number: B252

The manner by which dynactin regulates mammalian cytoplasmic dynein is dependent on the dynein intermediate chain isoforms.

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Cytoplasmic dynein is a motor protein responsible for the movement of membrane bounded organelles and other cargo toward microtubule minus ends. The dynactin complex is associated with several of dynein's functional properties. We previously reported that specific phosphorylation of the dynein intermediate chain isoforms (IC) was important for dynein binding to endosomes and that this phosphorylation did not modulate IC binding to the p150 subunit of dynactin. We therefore sought to directly examine the functional significance of the interaction of dynactin and dynein complexes for dynein motility in axons. We disrupted the interaction of dynactin and cytoplasmic dynein by over-expressing the first coiled-coil region (CC1) of the p150 subunit of dynactin in neurons. This domain competes with endogenous p150 for binding to the dynein IC, but does not dissociate the dynactin complex. Using live cell imaging of axons expressing fluorescent-tagged ICs, we found that there was significantly less movement of dynein containing IC-2C in the presence of CC1 than in the control. The motility of dynein containing IC-2C was significantly reduced in both directions of movement, 40% in the anterograde direction and 70% in the retrograde. These results are consistent with previous observations that disruption of the dynein-dynactin interaction reduces organelle motility. Importantly, we also observed that there was no inhibition of the motility of dynein complexes containing IC-1B in

either direction. We then examined the motility of two dynein cargoes. In the presence of CC1 we observed reduced motility of mitochondria, which we previously reported were transported by IC-2C containing dynein, but no inhibition of the motility of Rab7 containing endosomes, a cargo transported by IC-1B containing dynein. In the presence of CC1, we also observed a 33% decrease in run length for IC-2C dynein, but no effect on IC-1B dynein run length. These results demonstrate that when cytoplasmic dynein is detached from the dynactin complex *in vivo*, there are two distinct dynein motility responses which are correlated with specific dynein intermediate chain isoforms. The disruption of dynactin binding has minimal effects on the motility of dynein containing IC-1B or its cargo, while the disruption significantly inhibits the motility of dynein containing IC-2C and its cargo. Furthermore, the data strongly suggest that the interaction between the IC and p150 is not required for dynein containing IC-1B to bind to cargo, and that dynein containing IC-1B does not require signals transmitted through the intact dynactin complex (via p150) for normal motility. These data have important implications for studies of dynein populations that have mixtures of IC isoforms.

Microtubule Nucleation and Organization

P1736

Board Number: B254

Microtubule asters grow to span a millimeter-sized egg by nucleation remote from centrosomes.

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The large cells in early vertebrate development face an extreme physical challenge in organizing their cytoplasm. For example, frog embryos are 1.2 mm in diameter but have to divide every 30 min. In these cells, radial arrays of microtubules called asters grow, interact and move to precisely position the cleavage plane. Although the rapid expansion of asters, spanning the entire cytoplasm, is required for cells to explore its size and shape, it is unknown how microtubules assemble at such large distances from their presumable nucleation centers or centrosomes. Here, we combine quantitative imaging and cell-free reconstitution to show that microtubule asters grow by microtubule nucleation away from the centrosome. Aster growth occurs in the absence of microtubule transport while plus ends undergo both polymerization and depolymerization. Further, we report that the embryonic interphase cytoplasm supports spontaneous microtubule assembly independent of centrosomes, consistent with previous reports *in vivo*. We propose that aster growth, initiated by the centrosome, is supported by microtubule nucleation and is stimulated by the presence of pre-existing microtubules.

P1737**Board Number: B255****A new microtubule end-binding protein, NOCA-1, coordinates with patronin to control assembly of non-centrosomal microtubule arrays in *C. elegans*.**S. Wang¹, K. Oegema¹, A. Desai¹;¹Ludwig Institute for Cancer Research, La Jolla, CA

In contrast to the centrosomal microtubule arrays in dividing cells, many differentiated cells assemble non-centrosomal microtubule arrays adapted for specific cellular functions. In an RNAi screen in *C. elegans*, we identified NOCA-1 as a novel protein that does not contribute to centrosome-driven embryonic cell divisions but is required to form microtubule arrays in the germline that are essential for organismal fertility. The *noca-1* gene encodes 8 isoforms expressed in a variety of tissues. Two distinct long isoforms control assembly of microtubule arrays in the germline and embryonic epidermis, respectively. In contrast, a short isoform functions in parallel to the microtubule minus end-binding protein Patronin (PTRN-1) to control assembly of microtubule arrays in the post-embryonic epidermis. Evidence for the redundant activity of NOCA-1 and PTRN-1 includes synthetic lethality and early larval stage dye permeability of *noca-1Δ;ptrn-1Δ* double mutants, both of which are rescued by selectively expressing PTRN-1 in the post-embryonic epidermis. In support of the genetic interaction, NOCA-1 co-sediments with taxol-stabilized microtubules from worm lysates and purified recombinant NOCA-1, like PTRN-1, binds to microtubule ends in a microtubule-anchoring assay. Live imaging of the epidermal syncytium where NOCA-1 and PTRN-1 function redundantly revealed an array of evenly spaced microtubule bundles that run circumferentially around the animal. This array, comprised of both stable and dynamic microtubules, is subtly affected in single *noca-1Δ* and *ptrn-1Δ* mutants but nearly completely eliminated in *noca-1Δ;ptrn-1Δ* worms, suggesting that this specialized non-centrosomal array supports epidermal integrity during animal growth. We conclude that NOCA-1 represents a new class of microtubule end-binding proteins with essential functions of its own, as well as parallel functions with Patronin, in the assembly of non-centrosomal microtubule arrays in multiple *C. elegans* tissues.

P1738**Board Number: B256****A Detailed Centrosome Interactome Reveals a Novel Role for Asterless/CEP152 in Controlling Centriole Length Via Cep97.**B.J. Galletta¹, K.C. Jacobs², C.J. Fagerstron², K.C. Slep³, N.M. Rusan¹;¹National Heart Lung, and Blood Institute, NIH, Bethesda, MD, ²NHLBI-NIH, Bethesda, MD, ³Department of Biology, University of North Carolina, Chapel Hill, NC

A critical gap in our understanding of how centrosomes are built and subsequently mature into microtubule organizing centers (MTOC) is the relative lack of information on the direct interactions

among the constituent proteins. To address this deficiency in our knowledge, we performed a directed yeast two-hybrid screen to identify the possible, direct interactions among 22 conserved *Drosophila* centrosome proteins. To provide additional detail about how centrosomal proteins interact, these 22 proteins were subdivided into smaller fragments, keeping known domains and predicted secondary structures intact. In total, 67 full-length proteins and subfragments were tested. We identified more than 100 interactions among these proteins, the majority of which are novel, significantly increasing our understanding of the protein-protein interaction network within the centrosome. With this new interaction information, we attempted to better understand the role of Asterless (Asl)/CEP152. Since Asl is essential for the duplication of the centrosome, the molecular mechanism of its function and if it plays roles in addition to centriole duplication remains somewhat unclear. We have studied the loss of *asl* in the male germline stem cells (mGSCs) of *Drosophila melanogaster* larvae, which reproducibly harbor Asl-free centrioles. These remnant centrioles were built during embryogenesis using maternally provided Asl, but by this stage no longer have any associated Asl protein. Amazingly, in the mGSCs of *asl* mutants, the remnant centrioles are 10 to 30 fold longer than in wild-type mGSCs. Examining the localization of all of the Asl interacting proteins identified in our screen, we found proteins that localize to the proximal end of the giant centrioles in wild type spermatocytes extend the entire length of the centriole in the mGSCs of *asl* mutants. Interesting, the localization of proteins along the long centrioles in *asl* mGSC appears unique in comparison to previously described long centrioles, suggesting they may arise by a novel mechanism. To understand the possible mechanism of centriole length control by Asl in mGSCs, we examined flies with a mutation in *cep97*, which has been suggested to regulate centriole length. These flies have long centrioles in mGSCs, similar to Asterless. This is consistent with a model where in mGSCs, Asl plays a role in controlling the length of centrioles via Cep97. To test this model we have engineered a centriole-targeted version of Cep97 and show that expression of this construct can partially rescue the length of centriole in *asl* mGSCs. Finally, we are exploring the consequences of loss of Asl and the resulting increased length of centrioles on the interphase positioning of centrioles in mGSCs.

P1739

Board Number: B257

From Yeast to Human: Kinesin Regulators of γ -TuRC Structure and Nucleation.

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Members of two conserved mitotic kinesin-like protein (Klp) families, Kinesin-14 and Kinesin-5, have emerged as direct regulators of the γ -tubulin ring complex (γ -TuRC) microtubule organizing center (MTOC) in fission yeast and humans. While Klp members in these families are most studied for microtubule based organization roles in spindle assembly, a subset of members also localize to γ -TuRC at spindle poles to regulate microtubule nucleation. Genetic analysis, biochemical sedimentation of γ -TuRC complexes, co-immunoprecipitation assays and in vivo cell studies reveal a competitive mechanism between Kinesin-5 and Kinesin-14 that balances γ -TuRC structure and nucleation capacity.

We recently investigated details of the Kinesin-14 Pkl1 mechanism in fission yeast and the contribution of Kinesin-5 Cut7 to perturb the functional relationship between Kinesin-14 Pkl1 and γ -TuRC. We demonstrate that similar but counteracting forces by Kinesin-5 on γ -TuRC interfere with Pkl1 Motor and Tail domain interactions at γ -TuRC. We designed multiple unique Pkl1 Tail peptides as tools for capturing γ -TuRC or disrupting its structure by removal of γ -tubulin subunits (Cell Cycle 2013). This action is reversible and also occurs on γ -TuRC of rat and human centrosomes. New data from our lab further indicates that the normally essential fission yeast Kinesin-5 Cut7 motor protein becomes dispensable in the absence of Pkl1, emphasizing the importance of this antagonistic MTOC regulatory relationship. In yeast the action is asymmetric and additionally influences spindle microtubule numbers and microtubule overlap. We demonstrate by in vivo studies in human breast cancer cell lines that similar to as in yeast our Kinesin-14 peptides block microtubule nucleation from γ -TuRC. Previously we demonstrated in vivo that human Kinesin-14 HSET, but not Drosophila Ncd, functionally replaces Kinesin-14 Pkl1 in fission yeast and more recently that the genes encoding human γ -TuSC proteins GCP2 and GCP3 fully replace fission yeast genes for the analogous Alp4 and Alp6 MTOC proteins (JCS 2013). Our research highlights conserved in vivo regulation of γ -TuRC that informs on its structure and capacity for nucleation and reveals novel underlying mechanisms.

P1740

Board Number: B258

In vitro reconstitution of efficient microtubule formation by the combined action of TPX2 and chTOG.

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Spindle assembly relies on a number of microtubule-associated proteins that contribute to microtubule organisation through microtubule crosslinking and transport, regulate microtubule dynamics and/or facilitate microtubule nucleation. TPX2 is a multi-functional microtubule-binding protein required for proper spindle formation in all higher eukaryotes. It is one of the key targets of the Ran pathway that is essential for chromatin-dependent microtubule assembly in *Xenopus laevis* egg extracts. Total spindle microtubule mass in *Xenopus laevis* is additionally controlled by the processive microtubule polymerases of the XMAP215/Dis1 protein family. The molecular mechanism of how these two proteins contribute to microtubule formation and whether they directly facilitate the nucleation process is not understood. Here we use a combination of microscopy-based in vitro reconstitution assays to elucidate the respective roles of human TPX2 and chTOG, the human ortholog of XMAP215/Dis1, in microtubule assembly. TPX2 is preferentially recruited to the microtubule lattice at growing microtubule ends by its central domain, where it prevents microtubule catastrophes without affecting microtubule growth rate. These properties are markedly different from the microtubule growth-promoting activity of the chTOG proteins. The microtubule stabilising effect of TPX2 is directly related to its function in microtubule nucleation: TPX2 also stabilises early sheet-like nucleation intermediates at the initial stages of microtubule assembly. Efficient tube formation and sheet closure, however, require the additional

presence of the microtubule polymerising activity of chTOG. Together, our results demonstrate that a combination of very distinct activities of two microtubule binders is both necessary and sufficient for efficient microtubule formation in solution. Furthermore, these findings provide important insight into the mechanism of microtubule nucleation stimulated by the Ran pathway during spindle assembly.

P1741

Board Number: B259

Genetic analysis of microtubule array organization in *Arabidopsis* hypocotyls.

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Microtubules (MTs) organize through time and in space to affect critical processes such as cell division, morphogenetic events, and vesicle trafficking. Flowering plants produce distinct MT array patterns in the absence of centrosomes or defined MT-organizing centers. Highly dynamic cortical microtubule arrays are proposed to influence cell morphology through alignment of cellulose microfibrils in the cell wall. However, the mechanisms controlling the organizational states of these MT arrays remain largely unknown. Cortical MTs in *Arabidopsis* hypocotyl cells nucleate from γ -tubulin complexes distributed throughout the plasma membrane and remain laterally attached to the membrane. Redistribution of polymer via treadmilling leads to MT-MT interactions and formation of higher order structures such as MT bundles. We have used plant hormones to synchronously induce hypocotyl cells to reorganize the cortical MTs into a transverse co-aligned array on a 2 hr time scale. Reorganization initiates at the cell midzone and progresses bidirectionally to the apical and basal ends of the cell without transiting through an obligate intermediate pattern. To determine the genes required for this MT array patterning event, we initiated a forward genetic screen in *Arabidopsis*. EMS-mutagenized seedlings were screened for hypocotyl growth defects, including reduced hypocotyl length, radial cell expansion, and twisting cell files, and candidate mutants were rescreened for defects in hormone-induced transverse MT patterning. A systematic quantitative evaluation of cell shape and MT array pattern coupled with time-lapse imaging and supersensitivity tests for MT disrupting drugs is providing data for clustering phenotypes into distinct groups for complementation and epistasis tests. Five phenotypic groups have been initially identified with one group verified as new katanin (MT severing protein) alleles. A temperature-sensitive mutant class displays a novel radial-like MT array (termed pinwheel) with the focal point at the midpoint of the outer periclinal cell face. Pinwheel mutants fail to reorganize MT arrays to the transverse co-alignment after hormone treatment and exhibit reduced axial hypocotyl growth following long-term exposure to hormones. Outcomes from the screen and analysis of pinwheel mutants will be presented.

P1742**Board Number: B260****Linker Scanning Mutagenesis of Yeast Microtubule Nucleating Components Spc97 and Spc98.**K. Fong¹, J. Tien¹, C. Payen², A. Zelter¹, B. Graczyk¹, M. Dunham², T. Davis¹;¹Department of Biochemistry, University of Washington, Seattle, WA, ²Department of Genome Sciences, University of Washington, Seattle, WA

The mitotic spindle is a large structure responsible for the regulation and organization of chromosome segregation. Misregulation of chromosome segregation can lead to aneuploidy or genomic instability. Microtubules play several key roles during mitosis—mediating chromosomal movements, stabilizing spindle structure, and positioning the spindle within the cell. The γ -tubulin small complex is a highly conserved complex, essential for microtubule nucleation in all organisms. In budding yeast, the γ -tubulin small complex is localized to the spindle pole bodies and is comprised of two γ -tubulin molecules and one copy each of Spc97 and Spc98. Despite our advances in understanding microtubule function, dynamics and nucleation, purified γ -tubulin complexes fail to efficiently recapitulate nucleation *in vitro*, suggesting that additional regulatory processes activate the nucleating complex. Based on EM structure, we hypothesize that Spc98 undergoes a conformational change that enables the γ -tubulin small complex to serve as a template for microtubule growth. We have performed a saturating linker-scanning mutagenesis to define the regions of Spc97 and Spc98 required for the small complex to function *in vivo* and promote the formation of a microtubule. *In vitro* transposition reactions generated a library of random 15 base pair insertions in each gene, with approximately 90% coverage. This extensive transposition library was collected and transformed into yeast to screen for viability and conditional lethality using a plasmid shuffle. Mutants that abolish function identify the essential regions of the protein and mutants that are temperature sensitive can be studied after shifts to restrictive temperatures to examine nucleation activity. Over 50,000 yeast colonies were screened and 4% of the colonies contained lethal mutations and 0.02% contained temperature-sensitive mutations. Sites of lethal insertions were identified and mapped by high-throughput sequencing. Representative lethal mutations have been studied to determine the effects on protein expression, localization, and small complex formation. Temperature sensitive mutants were inspected by fluorescence microscopy and *in vitro* nucleation assays to reveal several nucleation hyperactive and deficient mutants. When combined with high-throughput sequencing, linker-scanning mutagenesis was a powerful approach to identify essential regions of the γ -tubulin small complex and provide novel temperature-sensitive mutants with specific defects in microtubule nucleation.

P1743**Board Number: B261****MAP65 acts as a molecular yardstick to minimize microtubule overlap in Dictyostelium syncytia.**M.P. Koonce¹, K. Irizarry¹, I. Tikhonenko¹;¹Wadsworth Center, Albany, NY

The MAP65/Ase1/PRC1 family is known as bundling proteins important for managing regions of overlapping antiparallel microtubules during animal cell division and for crosslinking parallel and antiparallel microtubule arrangements during interphase in plants and fungi. Homodimers work in combination with kinesins and other factors to link adjacent microtubules in a compliant manner. We postulate here that the MAP65/Ase1/PRC1 family has an additional role in sensing and coordinating the elimination of antiparallel microtubule arrangements during interphase, a process important to maintain intranuclear spacing in syncytial cells. Dictyostelium cells often fail cytokinesis and form multinucleated cells that remain viable. A striking feature of these cells is that nuclei are generally dispersed in the cytosol and their associated microtubule arrays roughly occupy spatially distinct territories. Treatment of cells with nocodazole shrinks distances between centrosomes, indicating that microtubule length plays a role in array spacing, and suggesting that nuclear spacing is actively maintained. Dictyostelium contains two homologs of MAP65 (A,B); knockout of MAP65A results in a significant collapse of inter-centrosome distances and enhances microtubule array co-mingling. The average spacing between centrosomes in wild type binucleate cells is 7.2 μm , but is only 3.7 μm in the MAP65A knockout. Disruption of MAP65B accentuates cellular branching and formation of elongated microtubules. The overall growth rate of cells lacking either MAP65 isoform is similar to wild type. However, for the MAP65A knockout, there is a marked increase in multinucleated cells. We have previously shown that the interphase microtubule array in Dictyostelium is a highly dynamic structure whose position is influenced by dynein, Kinesin-4, and Kinesin-8 activities. Our current work indicates that MAP65 also plays a role in maintaining microtubule organization, in particular with separation of multiple interphase arrays in a common cytosol. Kinesins-4 and -8 are known to interact with MAP65/Ase1/PRC1 proteins and thus potentially could be targeted to microtubule overlaps to foster sliding or depolymerase activity that minimizes antiparallel microtubules. These roles would not only facilitate endomembrane management in complex environments, but also minimize the potential for misintegration of multiple centrosomes into the same nucleus during division. For this project, we are grateful to support from the National Science Foundation (IOS-1051612 and DBI-1062963).

P1744**Board Number: B262****Identifying Pathways Directing Plant Cortical Microtubule Array Organization.**D. Thoms¹, A.W. Elliott², R. Chaudhari¹, A.J. Hill², M.A. Kline¹, S.L. Shaw¹;¹Department of Biology, Indiana University, Bloomington, IN, ²Molecular and Cellular Biochemistry Department, Indiana University, Bloomington, IN

The interphase microtubules at the plant cell cortex play a defining role in plant cell morphogenesis. Cell shape arises through a combination of forces, but relies heavily upon the mechanical properties of the cell wall. Cortical microtubules aid in patterning the extracellular deposition of cellulose microfibrils, the major structural fiber impacting wall mechanics. Cells undergoing anisotropic growth typically have co-aligned microtubule arrays oriented transverse to the dominant growth axis suggesting that the anisotropic growth arises because of strongly oriented cellulose deposition. The molecular pathways that determine how the cell organizes microtubules into a transverse coaligned pattern have not been explicitly described. We discovered a hormone treatment that synchronously induces plant hypocotyl cells to form transverse coaligned microtubule arrays and resume anisotropic growth. To identify molecules involved in the related genetic pathways, a genetic screen is underway. Candidate mutants are classed based on statistical analysis of array pattern distributions, growth morphology, and chemical sensitivity to drugs targeting microtubule and cellulose synthase activity. Approximately 60,000 seedlings from 1350 mutant lines were screened, and 55 initial lines were identified as having a hypocotyl growth defect along with the inability to form transverse arrays. The majority of mutants show subtle hypocotyl growth phenotypes such as cells with slightly decreased cell anisotropy or twisting cell files. The most common phenotypes are found in mutants with disorganized, basket arrays, and normal cell anisotropy, but are hypersensitive to treatment with oryzalin. Other common phenotypes occur in mutants which form co-aligned array patterns other than transverse, but have significantly decreased cell anisotropy. Less common phenotypes are in mutants with dense and disorganized arrays, abnormal cell files and cell morphology, normal or slightly increased anisotropy, and varying sensitivities to oryzalin treatment. Rarer phenotypes are in mutants with a threefold increase in cell anisotropy in comparison to light grown wild type seedlings, and a novel array patterning consisting of multiple swirl-like regions of co-alignment in each cell. Single mutant phenotypes include an inefficient ability to bundle microtubules, a wispy microtubule defect with extreme oryzalin hypersensitivity, or a hypocotyl defect exhibiting projecting cell files when grown in 30°C with extreme oryzalin hypersensitivity. Future work includes full genomic sequencing to identify underlying mutations, epistasis tests, and localization studies of identified proteins during the formation of transverse arrays.

P1745**Board Number: B263****Paternal Poc1 is Essential for the Function of the Zygote Centrosome.**A. Khire¹, S. Hynek¹, E.D. Rodriguez¹, T. Avidor-Reiss¹;¹Biological Sciences, University of Toledo, Toledo, OH

Centrioles are conserved microtubule-based organelles that, together with pericentriolar material (PCM), form the centrosome. During oogenesis in animals, the centrioles are eliminated, and therefore, the ovum does not contribute centrioles to the zygote. Instead, centrioles are provided to the zygote by the sperm. However, compared to centrioles in somatic cells, in many animals, including *Drosophila*, the spermatozoa centriole has a different structure and/or protein composition. Recently, we showed that *Drosophila* spermatozoa provide, in addition to the giant centriole (GC), a centriole precursor, which we named the proximal centriole like (PCL). The PCL resembles a centriole precursor, but lacks the centriole hallmark of microtubules. In the zygote, the GC and PCL recruit maternally contributed PCM, and form a centrosome. This centrosome, which is found near the male pronucleus, forms astral microtubules that reach out to the female pronucleus. Then, the female pronucleus migrates along the astral microtubules until it fuses with the male pronucleus. Later, the GC and PCL duplicate and form the poles of the zygote's spindles. Here, we report that the centriolar protein, Poc1, plays a role in fertilization in *Drosophila*. Poc1 is a conserved centriolar protein that is essential for centriole elongation and centrosome function. In an EMS screen, we identified a missense point mutation in poc1 that effects the recruitment of Ana-1 to the PCL, but does not effect Ana-1 recruitment to the GC or the length of the GC in the sperm. Males with this mutation, which preferentially affects the sperm PCL, have reduced fertility. This reduced male fertility associates with two distinct phenotypes in the zygote: failure in female pronucleus migration and formation of monopolar spindles. These findings demonstrate that poc1 is a paternal affect mutation due to dysfunction of the zygotic centrosome. It also suggests that the PCL is essential for zygotic centrosome function.

P1746**Board Number: B264****A Chlamydia effector manipulates host microtubule dynamics to promote bacterial survival.**M. Dumoux¹, A. Menny¹, D. Delacour², R. Hayward¹;¹Biological sciences, Institute of structural and molecular biology (Birkbeck college-UCL), London, United Kingdom, ²Institut Jacques Monod - CNRS, Paris, France

Chlamydia trachomatis is an obligate intracellular bacterial pathogen that causes sexually transmitted disease worldwide and is the leading cause of acquired blindness (trachoma) in developing nations. Chlamydiae employ a type III secretion system to deliver virulence effector proteins into host cells. These effectors subvert fundamental cellular processes, allowing bacterial intracellular survival and replication within a specialized membrane-bound compartment termed an inclusion. We discovered

that *C. trachomatis* dramatically reorganizes the microtubule cytoskeleton within infected cells to form a filamentous superstructure. This comprises a microtubule scaffold surrounding the inclusion and an associated nest of microtubules that originate at the inclusion and extend towards the plasma membrane. Using a bioinformatics approach, we identified a chlamydial effector (inclusion protein acting on microtubules; IPAM) that shares limited sequence similarity with eukaryotic centrosomal and microtubule-binding proteins. Using pull-down and co-immunoprecipitation assays we demonstrate that IPAM, a hydrophobic effector known to associate with the inclusion membrane, interacts with host centrosomal protein 170kDa (CEP170), a centrosome and kinesin-interacting protein, via a domain exposed to the cytosol. Ectopic expression of this IPAM domain impairs microtubule dynamics. This defect was restored by simultaneous CEP170 knock-down, demonstrating functional interplay between IPAM and CEP170 in cells. CEP170 is essential for chlamydial control of host microtubule dynamics and organelle repositioning during infection, and is necessary for inclusion morphogenesis and bacterial infectivity. Moreover, as IPAM stimulates CEP170 functions in *Chlamydia*-infected cells, this reveals potential physiological roles of CEP170 repressed in non-dividing uninfected cells. Together our data show how a chlamydial type III secretion effector stimulates a host target to promote the reorganization of the microtubule cytoskeleton in infected cells, an event required for inclusion biogenesis and bacterial intracellular survival. In turn this provides broader insight into the control of cellular microtubule dynamics.

P1747

Board Number: B265

Role of osmotic pressure in the regulation of the cytoskeleton during mitosis.

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The actin cytoskeleton plays an important role in the regulation of many cellular activities, including cell motility and cell division. The flat, adherent cells during interphase, assume a spherical shape at the onset of mitosis, termed as “mitotic cell rounding”. This change in cell shape is essential for the proper positioning and stabilization of the mitotic spindle, thus ensuring chromosome segregation with high fidelity. The remodeling of the actin cytoskeleton and the ability of the cell to regulate its osmotic pressure are among the key forces that primarily govern this cell rounding. A balance between the outward directed osmotic pressure and the inward actomyosin contraction is instrumental in driving this process. The ERM (Ezrin, Radixin, Moesin) family of proteins, known to cross-link the plasma membrane with the underlying actin cortex, are associated with mitotic cell rounding. Previous reports suggest the ERM proteins, upon phosphorylation and activation during mitosis are redistributed towards the cell cortex, thereby contributing to the cortical rigidity. Our results show an upregulation in the p-ERM levels and increase in the cortical rigidity of the mitotic cells upon exposure to a hypertonic environment. The actin filaments in the cells undergo realignment and distribution to the spherical cortex during mitosis. The interaction of the cortical actin with the astral microtubules that radiate towards the cell cortex

from the spindle poles generates tensile forces, thus favoring proper spindle assembly. Exposure to the hypertonic stress resulted in defects in the astral microtubule arrangement and centrosome integrity. The impaired astral microtubule arrangement was restored to normal when the cells were recovered in an isotonic medium, following a hypertonic shock. We further observe that the increase in the astral microtubules is directly proportional to the duration of the hyperosmotic shock. Perturbation of the osmotic gradient also leads to a significant decrease in the length and width of the mitotic spindle, as well as shrinkage of the cortical actin. Thus, our studies suggest the existence of possible functional links between cytoskeletal regulators and proteins modulating the osmotic pressure in cells.

P1748

Board Number: B266

Bayesian analysis of FRET to quantitatively measure microtubule nucleation in vivo and in vitro.

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The mitotic spindle is a self-organizing structure that segregates chromosomes during cell division. The spindle is composed of microtubules, and microtubules are composed of the protein tubulin. Understanding microtubule nucleation is critical to understanding the mitotic spindle. Microtubule nucleation can be inferred from the temporal dependence of the amount of tubulin in polymer. We use FRET, a technique to probe protein-protein interactions, to measure the amount of tubulin in polymer in cell extracts. To detect FRET, we measure the lifetime of the excited state of the donor fluorophore, a technique commonly referred to as FLIM. We developed a Bayesian framework for analyzing FLIM measurements that rigorously accounts for all known experimental complications. This novel technique will allow us to quantitatively measure microtubule nucleation in situ and dissect the biophysics, biochemistry and cell biology of microtubule nucleation.

P1749

Board Number: B267

Mechanisms controlling the microtubule organizing center state of the cell.

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The centrosome is the major microtubule organizing center (MTOC) in dividing animal cells. In many types of differentiated cells, however, MTOC function is reassigned to non-centrosomal sites. We are using *C. elegans* intestinal cells as a model to study non-centrosomal MTOCs. The apical surfaces of intestinal cells become MTOCs during polarization: microtubule-nucleating proteins that are centrosome

components in dividing cells become localized apically and apical membranes nucleate the assembly of microtubules. Concurrently, the centrosome is turned off as an MTOC in polarized cells; centrosomes lose pericentriolar material, microtubule association, and microtubule nucleating capacity. Thus, intestinal cells switch from a centrosome MTOC during mitosis to an apical MTOC during polarization. A subset of intestinal cells divides following polarization. In these cells, the apical MTOC is removed and the centrosomal MTOC is reactivated, indicating that cells can switch in either direction between MTOC states but do not maintain both. To determine how the cell “chooses” its MTOC state, we tested dominance of MTOC states by fusing a polarized cell with a mitotic cell in developing embryos. Cell fusion experiments indicate that mitotic cytoplasm is capable of dictating the MTOC state: fusing a mitotic cell with a polarized cell results in the rapid loss of MTOC components from the apical surface and the growth of MTOCs at normally quiescent centrosomes. We are now pursuing the mechanism that “flips the switch” in cellular MTOC state and will present our results.

P1750

Board Number: B268

A Novel Proximal Centriole-Like (PCL) Structure Becomes the Second Centriole in the Zygote of *Drosophila melanogaster* .

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Centrioles are conserved organelles that form centrosomes, or microtubule organization centers that are essential for cell division and function. As a result, they are essential for fertilization, development, and physiological functions. During oogenesis in animals, centrioles are lost, and thus, the oocyte does not contribute any centrioles to the zygote, the first cell of the embryo. Instead, it has been reported that zygotic centrioles are inherited from the sperm. In animals like sea urchins, frogs, and *Caenorhabditis elegans*, the sperm provides two centrioles, which is the normal number of centrioles in a cell. In contrast, in humans and in most mammals, the sperm contributes only a single centriole. This reduced contribution is due to a process known as “centrosome reduction,” in which one of the spermatid’s two centrioles degenerates during the last phase of spermatogenesis. Thus, the origin of the second zygotic centriole is uncertain.

Like humans, *Drosophila melanogaster* sperm contains one centriole (Giant Centriole/GC). However, we have recently discovered that *Drosophila melanogaster* sperm contains a centriolar structure that is composed of centriolar proteins but lacks the structural characteristics of a centriole; we have named it the Proximal Centriole-Like (PCL). Based on the PCL’s presence, we propose the PCL Hypothesis, which states that the PCL is a second zygotic centriolar structure. Here we report that like the GC, the PCL also loses many of its components during the late stage of spermiogenesis. Because of centrosome reduction, centriolar proteins do not label the PCL in the sperm and therefore, we indirectly visualize the PCL and GC by their recruitment of centrosomal markers in the fertilized zygote. Immediately after fertilization, we find two centriolar structures, the GC and the PCL. As expected from the PCL hypothesis, we find that early maternally contributed centriolar proteins like Sas 4 and Sas-6, do not

label the first two-centriolar structures of the zygote, but do label their daughter centrioles. Lastly, as the PCL hypothesis predicts, we found that zygotes lacking Asterless, which is essential for centriole duplication, have two centriolar structures. Altogether, we have found that the PCL is a centriole precursor in the sperm, which functions as the second zygotic centriole in *Drosophila melanogaster*. The PCL hypothesis may be applicable to other animals; it would explain why only three centrioles have been observed in a mammalian zygote during mitosis, despite the requirement of four centrioles for normal mitosis. Further studies aim to identify the range of animals with a PCL, to identify the specific role of the PCL in the zygote, and to identify the molecular mechanism underlying PCL function

Ciliopathies

P1751

Board Number: B270

Deficiency of tuba, a cdc42 GEF, disrupts normal zebrafish ciliogenesis and kidney development.

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Background: Dysfunction of renal primary cilia leads to polycystic kidney disease (PKD). The exocyst, a highly-conserved eight-protein membrane trafficking complex, is essential for ciliogenesis, and is regulated by Rho and Rab family GTPases, such as cdc42. We previously showed that cdc42 deficiency disrupts renal ciliogenesis and causes PKD in zebrafish and mice, and that Tuba, a cdc42-specific GEF, is necessary for ciliogenesis in cultured renal tubular MDCK cells. **Methods:** To determine how tuba affects ciliogenesis and kidney function in vivo, tuba knock-down was performed in zebrafish by microinjection of a translation blocking morpholino (MO). The phenotypic and histological defects caused by tuba deficiency were analyzed using whole-mount in situ hybridization, H & E staining and immunofluorescence. **Results:** Tuba was expressed in several tissues containing primary cilia, including the brain, neuromast cells, and renal tubules. We found that tuba morphants phenocopied cdc42 morphants, with ciliary mutant phenotypes including: a curly tail, hydrocephalus, and abdominal fluid accumulation. These defects were rescued by human tuba mRNA, which was resistant to the morpholino due to a difference in primary base pair structure, indicating that the phenotype was due to tuba deficiency, and not off-target effects. In the tuba morphant kidney, pronephric cilia were short and disordered, and the glomeruli were also disorganized. Because tuba is a known GEF of cdc42, and tuba and cdc42 morphants shared the same phenotype, we next performed a genetic synergy experiment in which we knocked down both tuba and cdc42. Co-injection of tuba and cdc42 morpholinos at low concentrations, which individually resulted in no phenotype, caused a severe phenotype, suggesting that tuba and cdc42 act in the same pathway. **Conclusion:** Our study showed that tuba deficiency causes an abnormal renal ciliary phenotype in zebrafish, demonstrating that tuba plays a critical role in

ciliogenesis and kidney development in zebrafish. We are currently generating kidney-specific tuba knockout mice to confirm these results in metanephrogenesis.

P1752

Board Number: B271

The Na⁺/H⁺ Exchanger Regulatory Factor 1 (NHERF1) regulates Wnt signaling pathways.

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The Na⁺/H⁺ Exchanger Regulatory Factor (NHERF1) is a scaffolding protein of the PSD-95/Discs-large/Zo-1 (PDZ) family. NHERF1 contains two tandem PDZ domains through which it mediates protein-protein interactions. Mice that do not express NHERF1 present various pathological phenotypes including hydrocephalus associated to disorganized ependymal cilia and reduced number of ciliated cells. Our studies demonstrate that these phenotypes can be explained by deregulated Wnt signaling in the absence of NHERF1. We show that NHERF1 binds a subset of Frizzled receptors (Fzd) and inhibits activation of β -catenin. NHERF1^{-/-} animals have increased levels of nuclear β -catenin in the ependyma. We further demonstrate that NHERF1 binds the Planar Cell Polarity proteins Vangl1/2 and promotes their traffic to the plasma membrane, and in particular to the apical surface of ependymal cells. Furthermore, we show that NHERF1 assembles a ternary complex containing Fzd4 and Vangl2. We hypothesize that NHERF1 is a “signaling switch” that regulates the input to the canonical and non-canonical Wnt signaling pathways by promoting the assembly of multimolecular complexes containing Fzd receptors and PCP proteins.

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P1753

Board Number: B272

Functional studies of NPHP5, a novel centrosomal protein deficient in human ciliary diseases.

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The purpose of this project is to understand the regulatory mechanisms of centrosomes and cilia function. Centrosomes are small organelles composed of two centrioles surrounded by a pericentriolar

matrix. They are the major microtubule-organizing centers in mammalian cells and regulate diverse cellular processes such as cell division. In addition, centrosomes promote the formation of cilia, cellular protuberances on the surface of quiescent cells critical for signal transduction. Centrosome and cilia dysfunction are linked to a wide variety of human diseases such as retinal degeneration or polycystic kidney disease. I am studying the function of one novel centrosomal protein named NPHP5. First, mutations in NPHP5 have been identified in patients suffering from Senior-Løken syndrome or Leber congenital amaurosis; two diseases with renal and/or retinal failure. Second, NPHP5 mRNA is up-regulated in gastrointestinal cancer but down-regulated by p53 after DNA damage; and its depletion causes pronephric cysts in zebrafish embryos. Finally, NPHP5 interacts with another centrosomal protein involved in ciliary diseases, Cep290. Despite its importance in health and disease, the precise function of NPHP5 is not fully understood. Our hypothesis is that NPHP5 cooperates with Cep290 to modulate cilia function and that its dysfunction contributes to the development of ciliary diseases. Using indirect immunofluorescence, NPHP5 was shown to localize to the distal region of centrioles in interphase cells. I have also demonstrated by RNA interference, immunofluorescence and immunoblot that NPHP5 depletion inhibits the migration of centrosomes to the cell surface, an early step of cilia formation. Using mapping studies and immunoprecipitation experiments, it was determined that NPHP5 interaction with Cep290 via its C-terminal region is critical for ciliogenesis. During those mapping studies, I also showed that the Cep290- and CaM-binding domains of NPHP5 are separable. Both domains are also distinct from the centrosomal localization domain, which contains the coiled-coil motif. Furthermore, it was observed by immunofluorescence that NPHP5 interaction with CaM prevents its self-aggregation. It was also demonstrated that disease-causing mutations of NPHP5 lack binding to Cep290 and are mis-localized from centrosomes, thus rendering the resulting proteins non-functional. Finally, using a pharmacological approach to modulate downstream events in the ciliogenic pathway, I showed that cilia formation could be rescued in the absence of NPHP5. These studies will improve our understanding of the biology of centrosomes and cilia and can lead to the development of new diagnostic and therapeutic applications.

P1754

Board Number: B273

The coiled coil domain containing proteins CCDC39 and CCDC40 are required for assembly of the nexin-dynein regulatory complex and affect tubulin polyglutamylation.

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The proteins, CCDC39 and CCDC40, were identified first as causative mutations in primary ciliary dyskinesia patients (Antony *et al.*, 2013). In *Chlamydomonas*, the *pf7* and *pf8* alleles are causative mutations in CCDC40 and CCDC39, respectively, based on reversion and rescue experiments. The *Chlamydomonas* mutations result in short, paralyzed flagella in which a fraction of the outer doublets fall into the center of the axoneme by electron microscopy as was observed in the PCD patients. CCDC39 and CCDC40 were hypothesized to be part of the nexin-dynein regulatory complex (N-DRC) based on the

patient phenotypes. To test this hypothesis, we used sliding assays and immunoblots to N-DRC proteins in *Chlamydomonas*. Isolated wild-type axonemes incubated with 0.1mM ATP slide along their entire length after treatment with protease, but show no sliding with just 0.1mM ATP. Axonemes from *pf7* and *pf8* with 0.1mM ATP and no protease show splaying of the microtubules, but remain attached at the proximal end as has been observed for other N-DRC mutants (Bower *et al.*, 2013). By immunoblots, the CCDC39 protein is absent in isolated *pf7* and *pf8* axonemes. Immunoblots with antibodies to N-DRC proteins (DRC1, 2, 3, 4, 5, 7, and 11) show a reduction or absence in isolated single and double mutant axonemes (*pf7*, *pf8* and *pf7*; *pf8*), which demonstrate that the mutational loss of CCDC39 and 40 is associated with the loss/reduction of known N-DRC proteins. Several lines of evidence suggest that CCDC39 and CCDC40 play an additional and novel role in tubulin polyglutamylation. The *ssh1* allele was identified as a suppressor of mutants lacking dynein arms that have short flagella (LeDizet and Piperno, 1985). Surprisingly, *ssh1* did not act as a suppressor of the short flagellar length in *pf7* and *pf8* strains, but further reduced flagellar length. The *ssh1* allele is a mutant in *TPG2*, which is needed for localization of the TLL9/TPG1 polyglutamylase (Kubo *et al.*, 2013; 2014). By electron microscopy, a fraction of the B-tubules fail to close properly in *pf7* and *pf8* axonemes. This phenotype is observed previously in various mutants that reduce polyglutamylation. Supporting these two lines of evidence, mutants in CCDC39 and 40 show a significant reduction in tubulin polyglutamylation in isolated axonemes by immunoblots. These results suggest that CCDC39 and 40 may function or alter the tubulin polyglutamylation pathway. We are using a temperature-sensitive *ccdc39* allele identified by whole genome sequencing to investigate the effects of loss of CCDC39 on IFT and the TPG proteins during temperature shifts and in dikaryons. In summary, CCDC39 and CCDC40 play roles in assembly of the N-DRC and in interactions with the tubulin polyglutamylation pathway

P1755

Board Number: B274

The human ciliopathy protein Jbts17 is required for basal body docking and intraflagellar transport.

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Ciliopathies are a broad class of human disease with a common etiology involving defective cilia structure or function. *Jbts17* (C5orf42) is a recently identified gene that has been found to be mutated in human patients with Joubert, Meckel-Gruber, and Oral-Facial-Digital Type VI syndromes, but its cellular and molecular functions have yet to be characterized. Here, we report the expression and functional analysis of *Jbts17* in *Xenopus* embryos and elucidate the pathogenic mechanism of human ciliopathy diseases associated with *Jbts17* mutations. We find that *Jbts17* is highly expressed in neural tissues and multiciliated cells (MCCs), and the expression of sonic hedgehog target genes, which is mediated through primary cilia function, is attenuated in *Jbts17* knockdown embryos. These embryos also exhibit several developmental abnormalities, including defective neural tube closure, eye formation, and

embryonic growth. We follow up by demonstrating that Jbts17 is strongly localized at basal bodies in MCCs and that Jbts17 knockdown cells are impaired in the capacity to dock basal bodies to the apical membrane. We also identified the specific domain required for Jbts17 basal body localization, which is truncated in some human ciliopathy patients. Finally, the normal expression and localization of Jbts17 at basal body is required for the basal body localization of both Inturned and Rsg1, proteins that are involved in basal body docking and intraflagellar transport. Our study provides direct evidence for the role of Jbts17 in ciliogenesis and the pathogenic mechanism of its associated human ciliopathy.

P1756

Board Number: B275

Cdc42 and sec10 are required for normal retinal development in zebrafish.

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Cilia are essential organelles required for a broad spectrum of functions, including left-right patterning, nephrogenesis, and vision. In the eye, development/maintenance/function of the photoreceptor requires transport of vesicles containing ciliary proteins from the Golgi to the outer segment by a modified cilium, called the connecting cilium. We previously demonstrated that the exocyst, a highly-conserved eight-protein complex, is required for renal ciliogenesis, most likely due to its role in targeting and docking vesicles carrying ciliary proteins. We also showed that Cdc42, a small GTPase, localizes with the exocyst at cilia, and biochemically interacts with Sec10, a crucial exocyst component. In this study, we examined the function of cdc42 and sec10 in eye development in zebrafish. Cdc42 and sec10 knockdown in zebrafish resulted in both abnormal eye development and increased retinal cell death. Cdc42 morphants had a relatively normal retinal structure, aside from the absence of most connecting cilia and outer segments, while in sec10 morphants, much of the outer nuclear layer, which is comprised of the cell bodies of photoreceptor cells, was missing and retinal-pigmented epithelial cell thickness was markedly irregular. Consistent with the proposed mechanism, knockdown of cdc42 and sec10 resulted in an intracellular transport defect affecting retrograde melanosome transport. Furthermore, there was a synergistic genetic interaction between zebrafish cdc42 and sec10, suggesting that cdc42 and sec10 act in the same pathway in retinal development. Based on these data, we propose a model whereby sec10 and cdc42 play a central role in trafficking ciliary proteins to the outer segment of photoreceptor cell for ciliogenesis and phototransduction.

P1757**Board Number: B276****HDACs and their role in Polycystic Kidney Disease.**P. Paul¹;¹Stowers Institute for Medical Research, Kansas City, MO

Autosomal-dominant polycystic kidney disease (ADPKD) is a monogenic renal disease characterized by progressive development of bilateral renal cysts, decline in renal function and many extrarenal effects. ADPKD has a very high worldwide prevalence rate of 1:400 to 1:1000 and it typically results in end-stage renal disease. ADPKD is caused by mutation in one of the two genes PKD1 and PKD2. PKD1 encodes polycystin-1 (PC1), which is a large G-protein coupled receptor-like protein and PKD2 encodes polycystin-2 (PC2), which is a TRP calcium channel. PC1 can interact with PC2 via its C-terminal coiled-coil domain to form a calcium permeable mechanosensor. Fluid flow on renal epithelium cell stimulates calcium influx, which activate kinases like protein kinase C. Our lab has showed that this kinase then directly or indirectly phosphorylates the 14-3-3 binding sites on histone deacetylase 5 (HDAC5) and causes nuclear export of HDAC5. This leads to the derepression of MEF2C target genes, one of which is missing in metastasis. It has been shown that pan-HDAC inhibitors can reduce cyst formation in PKD2^{-/-} mouse embryos supporting the important role HDACs play in ADPKD pathogenesis and thus in maintaining the normal organization of epithelial cells in kidney tubules. Further we confirmed that HDAC5 knockout can rescue polycystic kidney phenotype in adult PKD1 mouse model. Preliminary data suggest that even HDAC5 heterozygotes have significantly less percentage of cyst area and improved renal function compared to HDAC5 wild-type in a PKD1 deletion background. This further emphasizes the potential clinical implication of using specific HDAC inhibitors to reduce the progression of renal cyst in ADPKD patients. To better understand the mechanism of epigenetic regulation of cyst formation in ADPKD and to find novel therapeutic targets we wanted to understand the biochemical complex that HDAC5 forms in renal epithelium cells. HDAC5 forms complex with a class I HDAC, HDAC3 for its enzymatic activity. So a good therapeutic approach is to find specific inhibitors for HDAC3. To find drugs that can modulate the nuclear-cytoplasmic shuttling of HDAC5 we performed a screen, using a small pool of FDA approved compounds with well characterized targets involved in diverse signaling pathways using HDAC5 export as a paradigm. These studies together will give us mechanistic insights and also has the potential to identify novel therapeutic targets for ADPKD.

P1758**Board Number: B277****Loss of ciliary gene, Thm1, reduces expression of anorectic POMC, causing hyperphagia-induced obesity, fatty liver disease, diabetes, and hypertension.**D.T. Jacobs¹, M.P. Schonfeld¹, L.M. Silva¹, A. Chatterjee¹, G.C. Talbott², D.R. Beier³, P.V. Tran¹;¹Anatomy and Cell Biology, Univ Kansas Med Ctr, Kansas City, KS, ²Genetics Division, Brigham and Women's Hospital, Boston, MA, ³Center for Developmental Biology and Regenerative Medicine, Seattle Children's Research Institute, Seattle, WA

Obesity and metabolic syndrome are serious worldwide epidemics with treatments that are costly, invasive and largely ineffective. Primary cilia are antenna-like structures present on most vertebrate cells that mediate signaling pathways. Cilia defects cause ciliopathies, which manifest clinical features that are also common in the general population, such as obesity. The ciliary gene, Thm1, negatively regulates Hedgehog signaling and is most commonly mutated in patients with ciliopathies. We examined a postnatal role for murine Thm1 in obesity using a Thm1 conditional knock-out (cko) allele together with a ubiquitous, tamoxifen-inducible Cre recombinase. Thm1 deletion during adulthood caused a two-fold weight gain relative to control mice over a 13-week period. In adult Thm1 cko mice, adipose depots were significantly larger than in wild-type, and Thm1 cko livers had numerous, large lipid droplets, indicative of fatty liver disease. Results from serum glucose, leptin and insulin levels, as well as glucose tolerance tests indicate a diabetic state in Thm1 cko mice. Thm1 cko mice are also hypertensive relative to control mice. These observations indicate metabolic distress or 'metabolic syndrome'. To determine if the 19% increase in food intake observed in Thm1 cko mice caused the obese phenotype, Thm1 cko mice were pair-fed for 13 weeks following Thm1 deletion. Indeed, body weights and fat depots of pair-fed Thm1 cko mice were similar to controls, indicating hyperphagia is a primary cause of obesity. In the Thm1 cko hypothalamic arcuate nucleus, an integrative center for signals that regulate feeding and activity, we observed stunted primary cilia and reduced levels of anorectic pro-opiomelanocortin (POMC) transcripts. These data indicate that THM1 regulates response to satiety signals. To examine this role further, we are performing a genome-wide expression analysis on extracts of Thm1 cko arcuate nucleus using RNA sequencing. We are also examining the role of Thm1 in mouse hypothalamic cell lines using lentiviruses expressing Thm1 shRNA. Our data show that primary cilia are important in regulating energy homeostasis and provide an opportunity to identify therapeutic targets against the onset of obesity.

P1759**Board Number: B278****Loss of Occludin caused mucociliary clearance defect with reduced number of multiciliated cells in mouse.**E. Herawati¹, H. Tanaka¹, K. Tateishi¹, A. Tamura¹, S. Tsukita¹;¹Graduate School of Frontier Bioscience and Graduate School of Medicine, Osaka University, Osaka, Japan

This study aims at characterizing phenotype of mice lacking occludin, a transmembrane protein exclusively localized at tight junction strands, with particular reference to the respiratory system. In vivo data were acquired from previously generated Occludin knock out mice in trachea. On the other hand, in vitro data were collected from either wild-type or knock out derived mouse tracheal epithelial cells (mTEC) in culture. Occludin localizes at tight junction (TJ) in mTEC, the expression level of which was markedly increased in FOXJ1 positive cells until cells generated mature cilia. Occludin knock out mouse displays reduced number of the ciliated cells in trachea and accompanied by excess mucus plug in nasal cavity, suggesting a defect in mucociliary clearance function. Although the differentiation of trachea epithelial cells are normal, the detail analysis using electron microscopic images revealed that basal bodies of the ciliated cells were often incompletely docked. Further investigation is currently underway to examine the regulatory mechanism of how occludin is involved in maintaining normal ciliogenesis.

P1760**Board Number: B279****Repurposing of targeted cancer therapies for autosomal dominant polycystic kidney disease (ADPKD).**A.S. Nikonova¹, T. Seeger-Nukpezah², H. . Hensley¹, A. Klein-Szanto³, B. Egleston¹, E.A. Golemis¹;¹Developmental Therapeutics Program, Fox Chase Cancer Center, Philadelphia, PA, ²Department of Internal Medicine, Center of Integrated Oncology, University of Cologne, Cologne, Germany, ³Cancer Biology Program, Fox Chase Cancer Center, Philadelphia, PA

Autosomal dominant polycystic kidney disease (ADPKD) is an inherited progressive genetic disease that affects 1 in 500 people. With typical onset in middle age, with normal kidney tissue is gradually replaced with fluid-filled cysts resulting in end-stage renal disease for most patients. ADPKD is caused by mutational inactivation of polycystins 1 or 2 (PC1 and PC2, encoded by PKD1 and PKD2), which abnormally activates multiple signaling pathways regulating cell proliferation, migration, and response to environmental cues. A number of these signaling pathways are also activated in cancer, and targeted drugs are available to inhibit these pathways. There are currently no approved therapies to slow or cure ADPKD. We have been exploring inhibitors of Aurora-A, EGFR, and HSP90, using mouse models for ADPKD. We have previously reported that HSP90 inhibition with the small molecule STA-2842 in Pkd1^{-/-} mice reduces initial renal cyst formation and slows the progression of these phenotypes in mice with

pre-existing cysts. We now find similar effects in Pkd2^{-/-} mice. This is accompanied with defects in ciliogenesis and striking changes in signaling. In contrast, treatment of Pkd1^{-/-} mice with the Aurora-A kinase inhibitor MLN8237 (alisertib) increased the rate of cystogenesis in these mice. This was associated with defective ciliary resorption and morphological abnormalities of cilia. Intriguingly, while the EGFR inhibitor erlotinib had little independent effect on cystogenesis in Pkd1^{-/-} mice, combination of this agent with alisertib strikingly reduced alisertib-dependent promotion of cyst growth. The mechanistic basis for these signaling relationships will be discussed.

Ciliary/Flagellar Motility

P1761

Board Number: B280

Structural insights into the motility generation and regulation of cilia and flagella by cryo-electron tomography.

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Cilia and flagella play important roles in normal development and health of many eukaryotes, including humans [1, 2]. Their motility is driven by thousands of dynein motors [3, 4] that are coordinated by regulatory complexes, such as the Nexin-dynein regulatory complex and I1 dynein [5-8]. Our recent cryo-electron tomography study of intact sea urchin sperm flagella that were rapidly frozen while actively beating, revealed the 3D structures of dynein in three distinct post- and pre-powerstroke conformations [9]. These results provided important new insights into the structural basis of dynein's force generation [9], but how ciliary dyneins are regulated in detail and their different roles in generating ciliary motility remain unclear. Here, we analyzed cryo-tomograms of actively beating sea urchin sperm flagella to determine the 3D structures and conformational changes of all major regulatory complexes and dynein isoforms in relation to different regions of the sinusoidal wave of beating flagella (i.e., curved regions of principal or reverse bend, or straight regions between bends). For some of the dynein isoforms and predicted regulatory complexes we resolved distinct structures and distribution patterns of different conformations that correlate well with specific regions along the flagellar waves. Our comprehensive high-resolution analysis provides a new understanding of the molecular mechanisms underlying ciliary and flagellar motility and suggests a model that shifts previous hypotheses.

References: [1] Fliegauf et al. (2007) *Nat Rev Mol Cell Biol.* 8:880-93; [2] Mitchell (2007) *Adv Exp Med Biol.* 607:130-40; [3] Summers and Gibbons. (1971) *Proc. Nat. Acad. Sci. USA.* 68:3092-6; [4] Sale and Satir (1977) *Proc. Nat. Acad. Sci. USA.* 74:2045-9; [5] Huang et al. (1982) *Cell.* 28:115-24; [6] Piperno et al. (1994) *J Cell Biol.* 125:1109-17; [7] Bower et al. (2009) *Mol Biol Cell.* 20:3055-3063; [8] Toba et al. (2010) *Mol Biol Cell.* 22:342-53; [9] Lin et al. (2014) *Nat Cell Biol.* 16:479-85.

P1762**Board Number: B281****Single-molecule three-dimensional mapping of protein trafficking in primary cilia.**W. Luo¹, A. Ruba¹, W. Yang¹;¹Biology, Temple University, Philadelphia, PA

Primary cilia are a critical sensory organelle containing microtubule-based transport paths for proteins trafficking in cilium. Yet, the size of cilium (~ 300 nm in diameter) makes imaging three-dimensional (3D) transport routes for either membrane or soluble proteins inside live cilia still a major challenge. To localize protein trafficking in cilia, we employed a newly developed super-resolution microscopy imaging technique, termed single-point edge-excitation sub-diffraction (SPEED) microscopy, to real-time track various proteins in cilia with a spatiotemporal resolution of 5 nm and 2 ms in 3D. We found that both a membrane associated protein Arl13b and a transmembrane protein HTR6 possess spatially multiple distinct pathways in the primary cilium of *Odora* cells, agreeing well with a 9+2 microtubule pattern in *Odora* cilia identified by electron microscopy. Given the successful applications in mapping protein trafficking inside the cilia and the nuclear pore complexes, SPEED microscopy is proven to be an effective super-resolution high-speed imaging approach to map 3D tomography of macromolecules in sub-micrometer biocavities.

P1763**Board Number: B282****The asymmetric flagellar beat of *Chlamydomonas* corresponds to a sperm-like beat traveling around a semicircular arc.**V.F. Geyer¹, P. Sartori², F. Jülicher², J. Howard¹;¹Molecular Biophysics and Biochemistry, Yale University, New Haven, CT, ²Max Planck Institute for the Physics of Complex Systems, Dresden, Germany

Cilia and flagella are motile protrusions of eukaryotic cells that contain an evolutionary conserved internal structure, the axoneme. The axoneme is composed of a cylindrical arrangement of 9 doublet microtubules, transiently interconnected by dynein motors. Force generation by dyneins slides adjacent doublet microtubules. As a result of periodic sliding, the axoneme bends rhythmically, leading to its characteristic waveform. Interestingly, cilia and flagella generate two different types of waveforms, a symmetric, snake-like beat that pushes sperm through the surrounding fluid, and an asymmetric, breast-stroke-like beat that pulls biflagellated microorganisms such as *Chlamydomonas* through the fluid. The latter beat is similar to that of cilia that move fluid across epithelia. We have asked the question: what is the geometric difference between these different beating patterns? To answer this question, we have developed a simple, yet complete, shape characterization of the axonemal beat that can be used to compare waveforms both quantitatively and through theoretical modeling. We applied this approach to wild-type *Chlamydomonas* as well as to flagella from MBO2 mutants that have a symmetric beat. The

characterization uses Fourier analysis to decompose the periodic beat, expressed as tangent angle as a function of arc length and time, into a set of bending modes that include the time-averaged shape (“zeroth mode”), the waveform at the beat frequency (“first mode”) and higher order modes. We find that in both waveforms, the higher order modes make only a minor contribution. While the properties of the first order mode are similar in amplitude and phase, the zeroth modes are very different. While the MBO2 beat has a very small zeroth mode, corresponding to a mean shape that is almost straight, the wildtype beat has a much larger zeroth mode whose amplitude increases approximately linearly with arc length. This corresponds to a time-average angular shape that is a semicircular arc, around which the first mode propagates. We conclude that the dramatic differences between flagellar waveforms can be accounted for by differences in mean shapes.

P1764

Board Number: B283

Primary cilia bend and pivot in response to intracellular and extracellular forces.

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Primary cilia are ubiquitous, microtubule-based organelles which play diverse roles in sensory transduction in many eukaryotic cells. They interrogate the cellular environment through chemosensing, osmosensing and mechanosensing, using receptors and ion channels in the ciliary membrane. Little is known about the mechanical and structural properties of the cilium and how these properties contribute to ciliary perception. We probed the mechanical responses of primary cilia from kidney epithelial cells (MDCK-II), whose role it is to sense fluid flow in renal ducts. We found that upon manipulation with an optical trap, cilia deflect by bending along their length and by pivoting around an effective hinge located below the basal body. The calculated bending rigidity indicates weak microtubule doublet coupling. Primary cilia of MDCK cells lack inter-doublet dynein motors. Nevertheless we found that primary cilia display active motility. Three-dimensional tracking demonstrated correlated fluctuations of the cilium and basal body. These angular movements appeared random, but were dependent on ATP and cytoplasmic myosin-II in the cell cortex. We conclude that force generation by the actin cytoskeleton surrounding the basal body results in active ciliary movement. We speculate that actin-driven ciliary movement might tune and calibrate ciliary sensory functions.

P1765**Board Number: B284****IDA6 encodes a conserved subunit required for assembly of the N-DRC and several inner arm dyneins.**R. Bower¹, D. Tritschler¹, K. VanderWaal¹, E. O'Toole², T. Heuser³, D. Nicastro⁴, M.E. Porter¹;¹Genetics, Cell Biology, and Development, University of Minnesota Medical School, Minneapolis, MN,²MCD Biology, University of Colorado, Boulder, CO, ³Biology, Brandeis University, Waltham, MA,⁴Department of Biology, Rosenstiel Basic Medical Sciences Research Center, Brandeis University, Waltham, MA

The nexin-dynein regulatory complex (N-DRC) is a group of polypeptides thought to coordinate dynein activity and interconnect doublet microtubules (Piperno et al., 1994; Gardner et al., 1994; Heuser et al., 2009; Lin et al., 2012), but its precise role in flagellar motility is poorly understood. We have analyzed several *drc* mutants in *Chlamydomonas* to identify novel N-DRC subunits, to study N-DRC assembly, and to probe N-DRC function. To better understand the relationship between the N-DRC and the inner dynein arms, we characterized the *IDA6* gene. *ida6* is a motility mutant that fails to assemble dynein *e* and is associated with reduced levels of tektin, similar to the *drc* mutant, *pf3* (Kato et al., 1993; Gardner et al., 1994; Yanagisawa & Kamiya, 2004). Electron microscopy and 2D averaging revealed a major defect in assembly of the N-DRC, and so we tested candidates in the flagellar proteome (Li et al., 2004; Pazour et al., 2005) for rescue of *ida6* and identified the DRC2 subunit, FAP250, as the *IDA6* gene product (Austin-Tse et al. 2013). Mutations in the zebrafish and human orthologue CCDC65 are linked to defects in ciliary motility and primary ciliary dyskinesia. More recently we identified *sup-pf5* as another *drc2* mutation. DRC2 co-extracts, co-purifies, and co-IPs with other DRC subunits (Bower et al., 2013). iTRAQ labeling and MS/MS identified >10 axonemal proteins that are missing or reduced in *ida6* and *sup-pf-5*, and are restored in rescued strains. Spectral counting of dynein heavy chains (DHCs) revealed deficiencies in DHC8 and several other DHC isoforms. Cryo-electron tomography and 3D averaging further demonstrated that both *ida6* and *pf3* display similar defects in the assembly of the N-DRC and inner dynein arms, plus additional irregularities in the positioning of radial spokes. Thus *IDA6* encodes FAP250, which corresponds to DRC2, and DRC2 together with DRC1 plays a key role in assembly at the junction between the N-DRC, the radial spokes, and the dynein arms. Current studies focus on identifying the components that specify the binding sites for DRC1 and DRC2 on the outer doublet (Supported by NIH).

P1766**Board Number: B285****The ciliary N-DRC and B-tubule polyglutamylation are required for axoneme integrity and outer doublet alignment.**L.M. Alford¹, E.L. Hunter¹, M.E. Porter², W.S. Sale¹;¹Department of Cell Biology, Emory University School of Medicine, Atlanta, GA, ²Genetics, Cell Biology, and Development, University of Minnesota Medical School, Minneapolis, MN

The N-DRC is a large multi-subunit complex that interconnects adjacent doublet microtubules (Heuser et al., 2009). The N-DRC is predicted to limit microtubule sliding and to align outer doublet microtubules in the distal axoneme (Bower et al., 2013). To test this hypothesis, we examined ATP-induced reactivated motility of demembrated *drc* cells, commonly termed “reactivated cell models.” As described previously (Kamiya and Witman, 1984), ATP-induced reactivation of wild-type cell models resulted in the forward swimming of >90% cell models, similar to the movement of live cells. In contrast, ATP-induced reactivation failed in a subset of *drc*-cell models, despite obvious motility in live *drc*- cells. Dark field microscopic observations of *drc*-cell models revealed various degrees of axoneme splaying in *pf2*, *pf3*, *ida6*, *sup-pf3*, and *sup-pf5*. Immunofluorescence microscopy using an anti-tubulin antibody revealed that the distal axoneme was splayed in 60-85% of reactivated *drc*-mutant cell models, in contrast to wild-type cells in which >85% of axonemes remained intact. *sup-pf4*, unlike the other *drc*-mutants, retains most of the interdoublet linker domain of the N-DRC (Heuser et al., 2009). Notably, *sup-pf4* reactivated cell models displayed nearly wild-type levels of *in vitro* motility, and most *sup-pf4* and *sup-pf4*-rescued axonemes remained intact. These data strongly support the hypothesis that the N-DRC, particularly the interdoublet linker domain, is required for the integrity and interdoublet alignment of the distal axoneme. We also tested the idea that tubulin polyglutamylation plays a role in interdoublet interactions and axoneme integrity (see Kubo et al., 2012). Like the N-DRC mutants defective in the interdoublet linker domain, ATP-induced splaying of the distal axoneme was also observed in *tpg* mutant cell models. As proposed by Kubo et al., (2012), we postulate that the functional interaction of the N-DRC with the adjacent B-tubule is facilitated upon tubulin polyglutamylation.

P1767**Board Number: B286****Basal foot MTOC organizes pillar MTs required for coordination of beating cilia.**D. Clare¹, J. Magescas², T. Piolot³, M. Dumoux¹, C. Vesque⁴, E. Pichard⁵, T. Dang⁵, B. Duvauchelle⁵, F. Poirier⁵, D. Delacour²;¹Biological sciences, Institute of structural and molecular biology (Birkbeck college-UCL), London, United Kingdom, ²Institut Jacques Monod - CNRS, Paris, France, ³Curie Institute CNRS, Paris, France, ⁴INSERM, Laboratoire de Biologie du Développement, Paris, France, ⁵Institut Jacques Monod CNRS, Paris, France

Coordination of ciliary beating is essential to ensure mucus clearance in the airway tract. The orientation and synchronization of ciliary motion are controlled by the polarized organization of cilium bases at the

apical domain of epithelial cells, as illustrated by basal feet aligned in the same direction, and respond in part to the organization of the underlying cytoskeletal networks. Using dual-axis electron tomography procedure on mouse trachea, we show that microtubule (MT) and actin networks are independently organized in tracheal cells. On one hand, we observed sinuous actin microfilaments forming a dense meshwork encircling the basal bodies (BBs). On the other hand, the cortical cytoplasmic MTs originate from the BBs, mainly from the basal foot caps, where γ -tubulin is enriched. Most of the MTs are orientated obliquely and perpendicularly, with only a few that run parallel to the apical membrane connecting neighboring BBs. Then, BBs are collectively hooked at the cortex by a regular MT array composed of 4-5 MTs, and these cortical MTs may behave as cilium “pillars”. Removal of Galectin-3, one of basal foot cap components, provokes misrecruitment of γ -tubulin and disorganization of this cortical MT framework. Perturbation of the subciliary MT network at the base of the cilium had direct consequences on BB organization. The BBs were normally spaced but their alignment was impaired in *gal3*^{-/-} multiciliated cells, clearly showing that rotational cell polarity is disrupted in the absence of Galectin-3. Motile cilia do exist in *gal3*^{-/-} tracheas, but their structure and organization are affected, and reduced fluid flow is generated in mutants. Our data further emphasize the longstanding hypothesis that the basal foot cap is the main cilium base MicroTubule-Organizing Center (MTOC). In addition, we conclude that Galectin-3 plays a crucial role in the maintenance of the BB MTOC and the “pillar” MT units, and that this network is instrumental for the coordinated orientation and stabilization of motile cilia.

P1768

Board Number: B287

A spinning puzzle of the release of a giant multinucleate multiflagellate zoospore.

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Asexual reproduction in aquatic algal species of *Vaucheria* occurs by the formation of large multinucleate multiflagellate single cell. Termed as a zoospore, it is an elongated club-shaped zoosporangia at the tips of young branches. During development, the zoosporangia are separated from the rest of the thallus by membranes, resulting in multiple chambers hosting zoospores which will be released and dispersed in the surrounding aqueous environment. The apical gelatinization of the zoosporangial tip, together with the turgor pressure in the segregated portion of the filament, lead to a narrow aperture through which the zoospore escapes. However ordinary this may seem, *Vaucheria* zoospores have a unique multiflagellated patterned surface that warrants helicoidal flow entrainment at relatively high speeds, and which enables them to undergo a spinning motion that elastohydrodynamically assists the rather unfavorable escape maneuver. Experimental observations of this phenomenon, together with quantitative interpretations, are provided.

P1769**Board Number: B288****Structural analysis of the intraflagellar transport trains.**L. Stepanek¹, G. Pigino¹;¹Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany

Intraflagellar transport (IFT) is a non-vesicular transport pathway, which moves cargo proteins along the outer microtubule doublets of the axoneme. While primarily necessary for flagella/cilia assembly and maintenance, IFT also plays a role in cellular signaling and membrane trafficking.

The anterograde IFT trains move from the flagellar base to the tip, where they undergo rearrangement, possible cargo unloading and return in the form of retrograde IFT trains. Many aspects of these processes are poorly described, including the structural differences between the anterograde and retrograde particles. To characterize the differences in movement patterns of the two directions of transport I use total internal reflection fluorescence (TIRF) microscopy and single particle tracking techniques. To verify the results and to study the different morphology of the trains, I use TIRF correlated with transmission electron microscopy (TEM). The model organism of choice is biflagellate alga *Chlamydomonas reinhardtii*.

The results of particle tracking show that the IFT tracks are not evenly distributed throughout the cross-section of the flagellum of the gliding cells. Majority of the particles are moving along the lateral parts of the flagellum, indicating preference of some microtubule doublets as tracks for the IFT. To verify and refine these results, I use time-resolved correlative TIRF and TEM microscopy. Briefly, the IFT movement is stopped by fixative addition during time-lapse TIRF acquisition, so the directionality of the particles is known when the sample is imaged by TEM / TEM tomography. The high resolution of TEM is used to identify the microtubule doublets involved in IFT transport and to characterize the morphological difference between the anterograde and retrograde particles. This is the first time to show the structural properties of IFT trains related to their *in vivo* motility, opening further possibilities for more detailed studies of the IFT machinery.

P1770**Board Number: B289****Growth Arrest Specific 8 Mutant Mouse Reveals a Conserved Role for Motile Cilia Function.**W.R. Lewis¹, E.B. Malarkey¹, R.C. Pasek², J. Porath³, S.E. Birket⁴, S. Saunier⁵, C. Antignac⁶, M.R. Knowles⁷, M. Zariwala⁸, B. Kesterson⁹, S.M. Rowe⁴, K.F. Lechtreck¹⁰, I.A. Drummond¹¹, J.M. Parant¹², M.E. Porter¹³, F. Hildebrandt¹⁴, B.K. Yoder¹, N.F. Berbari¹⁵;

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Ciliopathies are genetic disorders of cilia dysfunction. These cellular appendages possess a microtubule based axoneme with either a doublet arrangement of 9+0 (primary/non-motile) or 9+2 (motile). Primary cilia have diverse sensory and signaling roles and as such, defects in primary cilia are associated with a wide spectrum of clinical features. Abnormalities in motile cilia result in Primary Ciliary Dyskinesia (PCD) which is characterized by chronic respiratory infections, situs inversus, infertility, and occasionally hydrocephalus. Here we demonstrate that mutations in the Growth Arrest Specific 8 (GAS8) gene, a putative homolog of Chlamydomonas Paralyzed Flagella 2 (PF2), cause PCD phenotypes in mice. We show Gas8 is required for proper assembly of the inner dynein arms (IDA) and the Nexin-Dynein Regulatory Complex (NDRC), and for normal cilia motility. Gas8^{GT} mice display abnormal cilia beat pattern and a lower beat frequency that Gas8^{WT} mice. Additionally, a screen for GAS8 mutations in PCD patients with situs defects identified two independent homozygous missense mutations whose pathogenicity is being confirmed utilizing model systems. The two independent mutations at c.595G>A and c.1172C>T interrupt a Gas8 Microtubule Association Domain (GMAD) and a Smoothened binding domain respectively. Currently, the pathogenicity of these human mutations is being tested in zebrafish and chlamydomonas. Utilizing CRISPR technology, we generated several Gas8 mutant zebrafish of which we will attempt rescue experiments with human WT, CT, and GA mRNA. Similarly, we are generating zebrafish with corresponding human mutations to analyze pathogenicity. Chlamydomonas is being used to investigate mechanistic defects associated with these human mutations by creating humanized mutations in PF2 and studying the effects on NDRC component docking. We hypothesize that both of these mutations will result in disruption of NDRC assembly and dyskinetic motile cilia.

Kinetochores Assembly and Functions 2

P1771

Board Number: B291

Measuring Force at the *Drosophila* Kinetochores.

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To maintain genomic stability, cells need to properly segregate their replicated chromosomes through cell division. As cells prepare to divide, a multi-protein structure called the kinetochores assembles at the

centromeres of each sister chromatid to mediate interactions with spindle microtubules. Proper chromosome segregation requires that microtubules emanating from opposing spindle poles bind sister kinetochores to form bioriented kinetochore-microtubule (kt-MT) attachments. Erroneous kinetochore kt-MT interactions lead to unequal chromosome segregation and aneuploidy. Therefore, kt-MT attachment stability must be carefully and tightly regulated during cell division to ensure the fidelity of the process. The application of opposing forces to kinetochores by dynamic MTs upon chromosome biorientation leads to a structural rearrangement of the kinetochore called intrakinetochore stretch in which the outer plate moves away from the inner plate. Since intrakinetochore stretch is an important regulator of kt-MT attachment stability, characterizing the properties of force-transducing kinetochore components and the magnitude of forces applied to these components are essential prerequisites for understanding how forces impact kinetochore structure and function. Existing measurements of the amount of force that is applied to kinetochores differ by orders of magnitude. To address this major discrepancy, calibrated force sensors were inserted into the *Drosophila* kinetochore. Since we acquired evidence that CENP-C is a central force-transducing component of the *Drosophila* kinetochore, two different calibrated force sensors were inserted into the middle of CENP-C to measure the force applied to bioriented kinetochores in living cells. One CENP-C reporter was built using a previously calibrated spider-silk-based FRET sensor and the second force reporter was made by inserting the rod domain of the focal adhesion protein Talin into CENP-C, and simultaneously expressing the head domain of Vinculin (a force-sensitive Talin binding partner) in the same cell line. Quantitative measurements of both reporters indicated that each CENP-C molecule is, on average, under 1 pN of force at bioriented kinetochores. Given that a *Drosophila* kinetochore has between 12-30 CENP-C molecules per MT that are envisioned as linkages arranged around the MT as a set of parallel springs, we propose that, on average, a kt-MT in *Drosophila* generates ~12-30 pN of poleward-directed force at metaphase. Furthermore, since there are 11 microtubules bound to *Drosophila* S2 cell kinetochores, we propose that each bioriented kinetochore, as a whole, experiences poleward-directed pulling forces between ~130 and 330 pN of force. It is noteworthy that this magnitude of force, in the hundreds of piconewtons range, differs significantly from recent measurements but is in close agreement with those initially measured by Bruce Nicklas in insect spermatocytes.

P1772

Board Number: B292

Mechanical Deformations Within Kinetochores During Chromosome Directional Instability.

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During mitosis, chromosomes move towards and away from spindle poles, and can undergo directional switches (Skibbens et al. 1993). These are driven by forces generated by the kinetochore, whose attached microtubules can switch between either polymerising or depolymerising states. These states

are associated with deformations within the kinetochore itself (Uchida et al. 2009, Dumont et al. 2012), and are also thought to respond to tension generated by the inter-sister linkage. To further understand these dynamics, we have recently advanced our kinetochore tracking assay (Jaqaman et al. 2010) in order to track eGFP-labelled CENP-A position at high temporal resolution (every 2s), which allows directional switch events to be carefully interrogated. We have also used Monte Carlo Markov chain methods to compare this data to a model of kinetochore dynamics, yielding information about forces within the system, providing new insight into the force-dependent mechanism that controls sister kinetochore switching. However, this does not incorporate kinetochore deformations, and therefore forces therein. Here I will present the evolution of our assay to simultaneously track mCherry-labelled Ndc80 position every 2s, thus allowing measurements of kinetochore deformations. Our initial analysis of inter- and intra-kinetochore distances yields key dynamic behaviour in the CENP-A-to-Ndc80 linkage both during continued directional motion and at directional switch events. We are also currently integrating these deformations in CENP-A-to-Ndc80 linkage into our modelling framework.

References:

Skibbens RV *et al.*, *Journal of Cell Biology* **122**, 859 (1993).

Uchida KSK *et al.*, *Journal of Cell Biology* **184**, 383 (2009).

Dumont S *et al.*, *Science* **337**, 355 (2012).

Jaqaman K *et al.*, *Journal of Cell Biology* **188**, 665 (2010).

P1773

Board Number: B293

Investigation of the *in vivo* mechanical properties of the kinetochore.

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The kinetochore is a macromolecular structure that is assembled on chromosomes during mitosis. The kinetochore provides the mechanical linkage between chromosomes and microtubules to translate microtubule dynamics into coordinated chromosome movement. As the sole connection of microtubules to chromosomes, kinetochores are essential to maintaining the fidelity of the genome during mitosis and, therefore, it is paramount to understand the mechanical properties of the kinetochore. In order to establish the *in vivo* mechanical properties of the kinetochore, I am using super-resolution live-cell imaging of chromosome movement upon depletion of kinetochore components. This assay is composed of acquiring spatially oversampled data followed by sub-pixel (super) resolution segmentation and tracking. I am using a strain of *C. elegans* expressing two GFP fusion proteins: GFP- γ -tubulin and GFP-histone H2B, allowing precise tracking and measurement of chromosome movements relative to the spindle poles (GFP- γ -tubulin). Each dataset is collected in 4D (x, y, z, t) to maximally capture chromosome movements from nuclear envelope breakdown through anaphase. In preliminary

experiments, I have achieved diffraction-limited 3D imaging at 100x 1.4NA with 3 second sampling. Each dataset is subjected to analysis using a custom Matlab program (celX) designed to automatically segment and track individual chromosomes in 4D. The output of celX manifests as numerous metrics of chromosome dynamics including, but not limited to, distance from the spindle axis, orientation of chromosomes relative to microtubules, and chromosome velocities. celX will allow me to track dynamic behaviors, such as oscillations or bending of chromosomes, with high spatial and temporal resolution. Chromosome movements are directly proportional to force imbalances, thus the tracking data can be transformed into relative force revealing the roles of individual kinetochore protein complexes. Initial RNAi screening of kinetochore components has focused on outer kinetochore complexes that are directly implicated in microtubule binding. Of particular interest is the KMN network that is proposed as the mechanical linkage between microtubules and kinetochores. The KMN network is composed of the Knl1, Mis12, and Ndc80 complexes and is the major player in end-on kinetochore-microtubule attachment. The measured velocities will provide insight into the mechanical properties of the kinetochore and begin to build a more comprehensive picture of the force landscape of chromosome movement. In sum, this analysis will provide accurate in vivo measurements of the movement and forces of chromosomes within the complex setting of the spindle.

P1774

Board Number: B294

Laser microsurgery reveals conserved viscoelastic behavior of the kinetochore.

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Accurate chromosome segregation depends on proper attachment of kinetochores to microtubules. Mounting evidence suggests that the mechanical properties of the kinetochore are fundamentally important for faithful segregation of chromosomes. We developed an assay where we use merotelic kinetochore as a model for studying mechanical properties of the kinetochore in vivo. A merotelic kinetochore is attached to microtubules emanating from both spindle poles and during anaphase, pulling forces exerted by microtubules lead to lateral stretching of such kinetochore. In our assay, we use laser ablations to sever microtubules attached to a stretched merotelic kinetochore, thus releasing the forces acting on this kinetochore. The mechanical properties of the kinetochore can then be inferred from the change of the kinetochore shape after microtubule severing. We used Hec1/Ndc80 and CenpA/Cnp1 fused to GFP to visualize outer and inner kinetochore domains, respectively, in mammalian

PtK1 cells and in the fission yeast *Schizosaccharomyces pombe*. In both model systems, kinetochores shortened after severing microtubules. An initial rapid shortening was followed by a period of slow relaxation. Interestingly, the inner kinetochore relaxed faster than the outer kinetochore. Whereas yeast kinetochores typically regained their unstretched size, many PtK1 kinetochores remained stretched over a longer period of time after microtubule severing. Our analysis of the time dependent kinetochore shortening reveals a viscoelastic behavior of the kinetochore that is evolutionarily conserved between yeast and mammalian cells.

P1775

Board Number: B295

Nanoscale patterning of the Ndc80 complex using a programmable DNA origami scaffold for architecture-function analysis of the kinetochore.

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The eukaryotic kinetochore is a macromolecular machine that uses multiple copies of many different proteins to mediate bidirectional chromosome movements during cell division. In budding yeast, three different microtubule-binding proteins: Ndc80, Dam1, and Stu2 are necessary for chromosome motility and their accurate segregation. In vivo studies also reveal that the yeast kinetochore incorporates a specific number of copies of each of these proteins: 8 copies of Ndc80, 16-20 copies of the of the Dam1 complex, and 7-8 Stu2 dimers. Moreover, these molecules are situated in a well-defined nanoscale organization at the microtubule plus end. This precise organization may be the key to achieving persistent kinetochore movement coupled to the polymerization and depolymerization of the microtubule. To directly test this hypothesis, we are developing an in vitro assay to pattern kinetochore proteins in pre-defined architectures and numbers. We have engineered a programmable DNA origami scaffold that provides up to six stable anchoring positions specified in an accurately defined geometry. Each position can be individually addressed so that either the number or the geometry or both can be varied to suit experimental design. Additionally, we are creating DNA origami scaffolds of different shapes and sizes to mimic/understand the kinetochore geometry at nanometer scale. We can achieve > 90% position occupancy with hybridizing DNA oligomers to the DNA origami scaffold as revealed by stepwise photobleaching using TIRF microscopy. We can attach recombinant Ndc80 complexes with a SNAP tag fused to the centromeric globular domain of the Ndc80 complex, and obtain homogenous populations of origami scaffolds for the given number of molecules as confirmed by gel-shift assays. Using this technique, we are systematically exploring the influence of the number and geometry of Ndc80 complexes on microtubule-binding and on generating persistent attachment. This technique provides a versatile and effective platform for a systematic study of the role of kinetochore architecture in defining the mechanism of kinetochore motility.

P1776**Board Number: B300****Chromosome segregation depends on a negatively-charged region of the β -tubulin carboxy-terminal tail.**C.P. Fees¹, J.K. Moore²;¹Univ Colorado Sch Med, Aurora, CO, ²Cell and Development, University of Colorado School of Medicine, Aurora, CO

Proper chromosome segregation requires carefully choreographed interactions between kinetochores and dynamic spindle microtubules. Whereas the roles of kinetochore proteins are relatively well understood, how tubulin proteins contribute to the fidelity of chromosome segregation is poorly understood. Here we investigate the negatively-charged carboxy-terminal tail (CTT) domains of the α - and β -tubulins, which are thought to promote electrostatic interactions with kinetochore proteins. CTT sequences are highly variable across species and tubulin isoforms, and are major sites of post-translational modifications. CTTs are, therefore, a possible point of regulating kinetochore-microtubule interactions that determine the fidelity of chromosome segregation. Using a series of mutants that alter or ablate CTTs of α - and β -tubulin in budding yeast, we identify a specific role for β -CTT in chromosome segregation. Mutant strains lacking the β -CTT exhibit delayed progression into anaphase and elevated rates of chromosome loss. In contrast, mutants lacking the α CTT appear similar to wild type. Using live cell imaging to measure the dynamics of kinetochores labeled with Nuf2-GFP, centromeres labeled with CENP-A/Cse4-GFP, and single centromeres labeled with CENIV-GFP, we show that loss of the β -CTT disrupts the bi-orientation of sister kinetochores. To elucidate the molecular role of the β -CTT, we map the residues that are necessary for function, and identify a short region of negatively-charged residues. Altering the charge of these residues disrupts chromosome segregation and microtubule dynamics. Furthermore, this negatively charged region may play an important role in facilitating interactions between the CTT and microtubule binding proteins. We provide evidence that this region supports the activity of the kinetochore protein, Ndc80. Based on these results, we propose that the β -CTT promotes proper chromosome segregation in two ways; by regulating the dynamics of spindle microtubules and by tuning kinetochore-microtubule interactions.

P1777**Board Number: B301****A Novel Role for the Rod-Zw10-Zwilch (RZZ) Complex in Modulating Microtubule (MT) Attachments at Kinetochores.**A.Z. Raja¹, J.M. Kasuboski¹, J. Kaneshiro¹, M. Joyce¹, W. Boggess¹, P.S. Vaughan¹, K.T. Vaughan¹;¹University of Notre Dame, Notre Dame, IN

Kinetochores utilize a combination of dynamic and stable MT attachments during the processes of chromosome alignment and chromosome segregation. Although phosphorylation is implicated in

regulating the MT-binding activities of some candidates, a mechanism that explains when each protein is dominant remains elusive. In previous work, we reported premature formation of stable MT attachments by HEC1/Ndc80 as a consequence of inhibiting Aurora B (AurB). This phenotype reflected the loss of the RZZ complex and all proteins dependent on RZZ, including cytoplasmic dynein, dynactin, spindly and MAD1/2. Testing a model in which RZZ masks the MT-binding activity of HEC1/Ndc80 until late stages of chromosome alignment, we sought additional kinases that regulate RZZ and identified Monopolar Spindle 1 (MPS1). MPS1 inhibition induced chromosome alignment defects including premature formation of stable MT-attachments. MPS1 inhibition also blocked recruitment of transient kinetochore proteins including RZZ and proteins that depended on RZZ. To define the requirements for MPS1 activity, we tested relevant candidates and identified Zwilch as a novel substrate for MPS1. MS/MS analysis revealed phosphorylation at three sites near the N-terminus of Zwilch and mutant analysis demonstrated that MPS1 phosphorylation is essential for RZZ function. 3A-mutant (T85A/S88A/S91A) Zwilch mimics MPS1 inhibition and blocks RZZ recruitment to kinetochores. 3E-mutant (T85E/S88E/S91E) Zwilch mediates recruitment of RZZ and RZZ-dependent proteins to kinetochores and renders cells resistant to MPS1 inhibition. Finally, phospho-mimetic Zwilch binds directly to Zw10, revealing a novel requirement for Zwilch phosphorylation in RZZ assembly. These findings support a model in which RZZ assembly and recruitment are responsible for modulating MT-attachments during prometaphase, perhaps through regulation of access to MTs by stable MT-binding proteins such as HEC1/Ndc80.

P1778

Board Number: B302

Ndc80 Tail Phosphorylation Controls Kinetochore-Microtubule Attachment Formation via Modulation of the Adjacent Calponin Homology (CH) Domain.

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The 4-subunit Ndc80 complex is a central component of the attachments between kinetochores and spindle microtubules that segregate chromosomes during cell division. The Ndc80 subunit of the complex has a conserved architecture with an unstructured N-terminal tail (N-tail) adjacent to a calponin homology (CH) domain. Although the N-tail is divergent in primary sequence between species, it harbors two conserved features: a highly basic nature (pI =10-12) and the presence of Aurora kinase target sites. Both the CH domain and N-tail have been proposed to make essential contributions to microtubule binding. The CH domain docks onto the microtubule lattice, while the basic N-tail is thought to promote binding through electrostatic interactions with the acidic C-terminal tails of α/β -tubulin. Correction of aberrant kinetochore-microtubule attachments requires Aurora B kinase, which is proposed to phosphorylate Ndc80 N-tails to counter their electrostatic interaction with the microtubule surface. Here we use the *C. elegans* embryo as a model system to test these ideas and examine the interplay between the CH domain, N-tail and Aurora B-mediated phosphorylation in controlling the formation of microtubule attachments. We show that, as expected, mutation of the CH domain prevents

the formation of load-bearing kinetochore-microtubule attachments *in vivo*. In contrast, whereas the N-tail is essential for microtubule binding *in vitro*, it is dispensable for both attachment formation and embryonic viability *in vivo*. Despite the fact that the N-tail is not essential for attachment formation *in vivo*, introducing phosphomimetic mutations into the 4 Aurora target sites in the N-tail perturbs attachment formation, phenocopying mutations in the CH domain. This observation suggests that rather than releasing an electrostatic interaction between the N-tail and the microtubule lattice, N-tail phosphorylation promotes attachment release by allowing the tail to function as an intra-molecular inhibitor of the CH domain. In support of this idea, synthetic genetic analysis in one-cell embryos revealed that the phosphomimetic N-tail resembles mutations in the CH domain instead of the N-tail deletion. These results lead to a new model in which Aurora kinase-mediated Ndc80 N-tail phosphorylation modulates activity of the adjacent CH domain to control kinetochore-microtubule attachment formation and ensure accurate chromosome segregation.

P1779

Board Number: B303

Spindle checkpoint protein Bub3 promotes mitotic cell cycle progression by activating APC/C-Cdc20 in budding yeast.

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The spindle checkpoint is a well-conserved surveillance mechanism, which functions at the metaphase-anaphase transition. In the presence of improperly attached kinetochore, the spindle checkpoint forms an effector complex to delay anaphase onset by binding and inhibiting Cdc20, the co-activator of APC/C (Anaphase Promoting Complex/Cyclosome). This effector complex is comprised of the spindle checkpoint proteins Bub3, Mad2 and Mad3. Here we show that deleting BUB3, but not MAD2, results in a prominent delay of anaphase onset. The delay is not a result of the other cell cycle checkpoints, or a result of aneuploidy. Surprisingly, co-immunoprecipitation studies show that Cdc20 binding to the APC/C complex is impaired in *bub3Δ* cells. In addition, the delay is also observed when Bub3 is prevented from localizing to the kinetochore. Bub3 and Cdc20 co-localize to the kinetochore in metaphase, indicating their interaction at the kinetochore could be important for timely anaphase onset. This study reveals the multifaceted function of the spindle checkpoint by illuminating a novel role of Bub3 in APC/C-Cdc20 modulation.

P1780**Board Number: B304****Mad1 and Bub1 – more than kinetochore recruitment factors in the spindle assembly checkpoint.**K. Sewart¹, N. Schmidt², S. Heinrich², M. Langeegger², S. Hauf^{3,4};¹Department of Biological Sciences, Virginia Tech, Blacksburg, VA, ²Friedrich Miescher Laboratory of the Max Planck Society, Tuebingen, Germany, ³Dept. of Biol. Sciences and VBI, Virginia Tech, Blacksburg, VA,⁴Friedrich Miescher Laboratory, Max Planck Society, Tuebingen, Germany

To maintain genome integrity, the genetic material has to be equally distributed during cell division. The spindle assembly checkpoint is a conserved surveillance mechanism that delays anaphase as long as any of the chromosomes is not properly attached to the mitotic spindle. Malfunction of this checkpoint causes erroneous chromosome segregation and has been implicated in tumorigenesis. Despite a wealth of information on the localization and interaction of checkpoint proteins, the *in vivo* signaling mechanism is still only partially understood.

A complex between the proteins Mad1 and Mad2 is crucial for the spindle assembly checkpoint. Kinetochore-bound Mad1:Mad2 binds a second molecule of Mad2 through Mad2:Mad2 dimerization, which is necessary for binding of this second Mad2 to the anaphase activator Cdc20. This ultimately prevents anaphase. Work of several labs, using different organisms, consistently showed that Bub1 is required for kinetochore recruitment of Mad1 and that kinetochore-bound Mad1 serves as binding platform for Mad2. Employing a combination of biochemical methods, yeast molecular genetics and live cell imaging approaches, we recently showed that the roles of Mad1 and Bub1 go beyond this role in bringing Mad2 to the kinetochore. We introduced mutations in the Mad1 C-terminus that preserved Mad1 kinetochore localization, its interaction with Mad2, and the capability for Mad2 dimerization, but nevertheless impaired checkpoint functionality. Similarly, artificial tethering of Mad1 to the kinetochore in cells depleted of Bub1 also did not rescue checkpoint functionality, although Mad2 was co-recruited with Mad1 and was able to dimerize. These results indicate that Mad1 and Bub1 are more than mere recruitment factors for Mad2. We are in the process of determining, which step, downstream of Mad2 dimerization, is defective and use the separation-of-function mutants of Mad1 to investigate the molecular role of the Mad1 C-terminus in this process.

P1781**Board Number: B305****Toward quantitative theoretical description of the kinetochore-microtubule interactions and their roles in ensuring accurate chromosome segregation.**A. Zaytsev¹, F.I. Ataulakhanov^{2,3}, E.L. Grishchuk¹;¹Physiology Department, University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA,²Center for Theoretical Problems of Physicochemical Pharmacology, Russian Academy of Sciences,Moscow, Russia, ³Physics Department, Moscow State University, Moscow, Russia

Mitotic chromosomes segregate equally between two daughter cells with the help of spindle microtubules (MTs). MTs attach dynamically to chromosomal kinetochores, but little is known about how molecular characteristics of kinetochore proteins contribute to the dynamicity and robustness of MT attachments, and how quantitative characteristics of these interactions affect the accuracy of chromosome segregation. Our long term goal is to use quantitative “bottom-up” modeling to link the molecular parameters of key kinetochore proteins with physiology of mitotic processes, seeking novel insights and predictions. To investigate chromosome segregation at different organizational scales we developed several theoretical models. The molecular-kinetic determinants of kinetochore MTs stability were examined using probabilistic single-molecule model of the MT binding at the kinetochore site with 12-20 molecules. We show that when these molecular interactions are independent and stochastic, the physiological rate of MT turnover is achieved only when the bindings are transient, each lasting fraction of a second (Zaytsev et al., CaMB 2013). MT bindings in vitro by the single molecules of the core kinetochore protein NDC80 last only 20-400 ms, so we next modeled the kinetochore interface using the experimentally determined NDC80 constants. We demonstrate that the stability of kinetochore MTs and its regulation during mitosis can be largely explained by the phosphoregulatable MT-binding affinity of just this one kinetochore component. Interestingly, the interactions between NDC80 complexes and kinetochore MTs are predicted to be unconstrained, meaning that the NDC80 molecules are able to alternate their binding between adjacent kinetochore MTs (Zaytsev et al., JCB 2014). Finally, we developed a probabilistic model for dynamic kinetochore-MT interface to systematically and quantitatively examine the impact of transient MT attachments on the accuracy of chromosome segregation. We show that the rate of kinetochore-MT turnover on its own has little impact on the final accuracy of MT attachments. Strikingly, a model that combines the optimal rate of MT turnover with the plate-like kinetochore geometry predicts the frequency of chromosome loss $(2.6 - 47) \times 10^{-2}$. The lower value is similar to what is seen in tumor-derived cells, but this simplified model cannot explain low chromosome loss frequency in normal human cells. Together, these theoretical approaches provide novel mechanistic insights into the molecular mechanisms of accurate chromosome segregation. This work should assist developing the comprehensive quantitative understanding of chromosome segregation in normal cells, and help to uncover how pathological chromosomal instability can be prevented.

P1782**Board Number: B306****Studying kinetochore-microtubule attachment using fluorescence lifetime imaging microscopy with Bayesian analysis.**T. Yoo¹, D.J. Needleman^{2,3,4};¹Harvard University, Cambridge, MA, ²FAS Center for Systems Biology, Harvard University, Cambridge, United States, ³School of Engineering and Applied Sciences, Harvard University, Cambridge, MA,⁴Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA

Accurate segregation of chromosomes requires the proper attachment of microtubules to chromosomes via the kinetochore. Kinetochore-microtubule attachments are thought to be predominately mediated by the Ndc80 complex, which is modified by Aurora B kinase, but the mechanism by which attachment is maintained and regulated is poorly understood. We are studying these processes with Bayesian analysis of Fluorescence Lifetime Imaging Microscopy (FLIM), a form of Forster Resonance Energy Transfer (FRET) capable of quantitatively measuring the dynamics of protein-protein interaction in cell with millisecond time resolution. We are investigating the dynamics of the binding and clustering of Ndc80 complexes on microtubules at kinetochores in human tissue culture cells. We are also studying phosphoregulation of kinetochore-microtubule attachment in cell by monitoring the activity of Aurora B kinase using a Aurora B biosensor.

P1783**Board Number: B307****A minimal model of kinetochore-microtubule dynamics captures metaphase oscillations, error-correction and effects of Aurora B hyperactivation.**K.K. Chiu¹, E.R. Ballister², E.J. Banigan³, A.M. Mayo², M.A. Lampson², A.J. Liu¹;¹Physics and Astronomy, University of Pennsylvania, Philadelphia, PA, ²Department of Biology, University of Pennsylvania, Philadelphia, PA, ³Physics and Astronomy, Northwestern University, Evanston, IL

Successful eukaryotic cell division requires the formation of correct attachments between kinetochores and spindle microtubules. In the correct (bioriented) configuration, sister kinetochores attach exclusively to microtubules from opposite spindle poles; these microtubules exert opposing poleward forces, resulting in inter-kinetochore tension. Kinetochore-microtubule interactions are dynamic and subject to tension-dependent regulation that destabilizes improper attachments. Tension acts directly via catch-bond-like effects and indirectly through Aurora B phosphorylation of kinetochore proteins. We developed a minimal stochastic model incorporating previous in-vitro biophysical measurements of kinetochore-microtubule interactions and their dependence on both tension and phosphorylation. These previous experiments used kinetochores that bind single microtubules; our model builds on these measurements to describe kinetochores that bind bundles of microtubules and qualitatively captures key phenomena including error correction and metaphase chromosome oscillations. We challenged this

model by experimentally manipulating kinetochore phosphorylation using chemically induced dimerization in living cells. Recruiting Aurora B to metaphase kinetochores caused a dramatic disorganization of the metaphase plate. Systematic analysis of our computational model showed that varying the simulated rates of microtubule catastrophe, rescue and detachment, but not other parameters, provided a good fit to experimental results. These are the same parameters which were independently shown to be modulated by Aurora B phosphorylation in vitro. These results establish a minimal physical model for kinetochore-microtubule dynamics that provides a framework for further theoretical development and quantitative interpretation of in vivo experiments.

P1784

Board Number: B308

Bi-stability of Aurora B kinase activity underlies spatial gradients in dividing cell.

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Aurora B kinase, the enzymatic component of the chromosome passenger complex, is a key regulator of cell division that provides temporal and spatial coordination for chromosome segregation. Gradients of Aurora B activity are implicated in regulating kinetochore-microtubule interactions and cytokinesis, but the mechanisms underlying these spatial patterns are unknown. Spatial phosphorylation gradients extend beyond known locations of Aurora B binding sites, suggesting that these patterns form by a Turing mechanism through dynamic self-organization of a coupled kinase-phosphatase system. Such a mechanism implies that the system contains positive feedback and exhibits bi-stability. To test the hypothesis that self-organization can establish spatial patterns of Aurora B activity, we use a coupled Aurora B kinase and phosphatase system in vitro, which is biochemically and theoretically tractable. We show that Aurora B kinase activation by auto-phosphorylation in trans leads to a bi-stable switch, and we experimentally define a hysteretic region for Aurora B activity that matches the theoretical prediction. In living cells, we find similar bi-stability using a FRET-based phosphorylation sensor: partial kinase inhibition leads to two distinct populations of mitotic cells with either high or low levels of Aurora B substrate phosphorylation. Together these results suggest that spatial patterns of Aurora B activity in cells are established around sites of CPC concentration by a Turing self-organization mechanism.

P1785**Board Number: B309****The spindle checkpoint components Mad1 and Mad2 have an essential, checkpoint-independent function in *C. elegans*.**P. Lara-Gonzalez¹, M. Moyle¹, T. Kim¹, D.K. Cheerambathur¹, K. Oegema¹, A. Desai¹;¹Ludwig Institute for Cancer Research, La Jolla, CA

The spindle assembly checkpoint promotes accurate chromosome segregation by restraining progression through mitosis until all the chromosomes are attached to the microtubules of the mitotic spindle. The most downstream components of this pathway are the “mitotic arrest deficient” (Mad) proteins Mad1, Mad2 and Mad3, which are conserved throughout evolution. While the spindle checkpoint and its components have been studied extensively from a single-cell perspective, we aimed to understand the consequences of disrupting this pathway in a developing organism, and ask whether the resulting phenotypes are explained by lack of checkpoint function. For this, we employed CRISPR-Cas9 to generate strains of *C. elegans* in which *mad-2* and *mad-3* were fully deleted; a full deletion of *mad-1* was previously generated. We found that, consistent with previous observations using partially deleted alleles, complete deletion of *mad-1* and *mad-2* has a profound effect on viability and fertility. Surprisingly, deletion of *mad-3* has only a mild effect on viability. Thus, despite all of the MAD proteins being essential for the spindle checkpoint, MAD-1 and MAD-2 have additional, checkpoint-independent roles, which are essential for organismal development. We confirmed this conclusion by analyzing mutations that disrupt the ability of MAD-1 to localize to unattached kinetochores; despite their lack of checkpoint activity, these mutant forms of MAD-1 supported normal viability and fertility. In addition, MAD-1 localizes to the nuclear envelope during interphase, but we engineered a mutant form of MAD-1 that abolishes this localization and found that it still supports normal viability and fertility. In contrast, a MAD-1 mutant that cannot interact with MAD-2 was phenotypically similar to a *mad-2* deletion, exhibiting severe loss of viability and fertility. These data indicate that the MAD-1/MAD-2 complex has an essential function during organismal development in *C. elegans*, which is unrelated to its known role in the spindle checkpoint. We are currently testing if the well-described MAD-1/MAD-2 complex-mediated control of the anaphase promoting complex/cyclosome (APC/C) underlies this essential function. In addition we are performing unbiased biochemical, molecular genetic, and developmental analyses to elucidate the non-checkpoint function of MAD-1/MAD-2 that is essential for organismal development.

P1786**Board Number: B310****The nanoscale organization of Spc105 in metaphase and its role in silencing the Spindle Assembly Checkpoint.**B. Roy¹, J. Sim¹, A.P. Joglekar^{1,2};¹Cell and Developmental Biology, University of Michigan, Ann Arbor, MI, ²Biophysics, University of Michigan, Ann Arbor, MI

Faithful chromosome segregation during cell division requires that the kinetochore, a multi-protein machine built on every chromosome, is stably attached to spindle microtubules. Whether or not the kinetochore is attached is monitored by a surveillance mechanism known as the Spindle Assembly Checkpoint (SAC). The SAC delays the cell cycle as long as even a single kinetochore is unattached. It gets silenced rapidly once stable attachments form. The physical platform for the SAC is provided by Spc105, a conserved kinetochore protein that is large protein (~ 900 amino acids) and consists of a large, unstructured amine-terminal (~ 500 amino acids) signaling domain with microtubule-binding activity. Upon phosphorylation by the Mps1 kinase, this domain recruits SAC proteins to activate the SAC. Just as the organization of microtubule-binding kinetochore proteins plays a key role in executing kinetochore movement, the organization of Spc105 in the attached kinetochore may be important for the reliable silencing of the SAC. Therefore, using in vivo Forster Resonance Energy transfer (FRET) and high resolution co-localization measurements, we defined the average localization and distribution of five different positions spanning the length of the Spc105 molecule. We find that the entire Spc105 molecule is compacted within a 30 nm zone bookended by the centromeric end of the Ndc80 complex (C terminus of Spc24) up to the C terminus of Ndc80 (subunit of the Ndc80 complex). Detectable FRET between Stu2, a microtubule plus-end binding protein, indicates that the N-terminus is proximal to the microtubule lattice. FRET between two different positions in the N-terminus of adjacent Spc105 molecules can also be detected, which is consistent with the disordered nature of this domain. This compact Spc105 organization is surprising, given its large size and disordered nature. We hypothesize that the microtubule-binding activity in Spc105 restrains it within the inner kinetochore in metaphase, and that this organization is required for reliable silencing of the SAC. We will test these hypotheses by abrogating the microtubule-binding activity of Spc105, and studying whether its nanoscale organization changes and if timely SAC silencing is impaired.

P1787**Board Number: B311****TRIP13 AAA-ATPase is a novel mitotic checkpoint silencing protein.**K. Wang¹, B. Sturt-Gillespie¹, J. Hittle², D. Macdonald³, G. Chan³, T.Y. Yen⁴, S. Liu¹;¹Biol Sci, University of Toledo, Toledo, OH, ²Fox Chase Cancer Center, Philadelphia, PA, ³Cross Cancer Inst, Edmonton, AB, ⁴Developmental Therapeutics Program, Fox Chase Cancer Center, Philadelphia, PA

The mitotic checkpoint (or spindle assembly checkpoint) is a fail-safe mechanism to prevent chromosome missegregation by delaying anaphase onset in the presence of defective kinetochore-microtubule attachment. The target of the checkpoint is the E3 ubiquitin ligase anaphase promoting complex/cyclosome (APC/C). Once all chromosomes are properly attached and bi-oriented at the metaphase plate, the checkpoint needs to be silenced. Previously we and others have reported that Thyroid hormone receptor interacting protein 13 (TRIP13) AAA-ATPase interacts with the mitotic checkpoint silencing protein p31comet. Here we show that endogenous TRIP13 localizes to kinetochores. TRIP13 knockdown delays metaphase-to-anaphase transition. The delay is caused by prolonged presence of the effector for the checkpoint, the mitotic checkpoint complex (MCC) and its association and inhibition of the APC/C. These results suggest that TRIP13 is a novel mitotic checkpoint silencing protein. The ATPase activity of TRIP13 is essential for its checkpoint function, and interference with TRIP13 abolished p31comet mediated mitotic checkpoint silencing. TRIP13 overexpression is a hallmark of cancer cells with chromosomal instability, particularly in certain breast cancers with poor prognosis. We suggest that premature mitotic checkpoint silencing triggered by TRIP13 overexpression may promote cancer development.

P1788**Board Number: B312****BUB-1 promotes the onset of anaphase independently of its role in the spindle checkpoint and chromosome alignment.**T. Kim¹, M. Moyle¹, C. De Groot¹, K. Oegema¹, A. Desai¹;¹Ludwig Institute for Cancer Research, La Jolla, CA

Bub1 is a conserved kinase implicated in spindle checkpoint signaling and chromosome alignment. Bub1 targets Mad1 to the kinetochore to initiate spindle checkpoint signaling. In addition, Bub1 promotes chromosome alignment by phosphorylating histone H2a to direct recruitment of the chromosomal passenger complex via the adaptor Shugoshin. Unexpectedly, we found that depletion of BUB-1 significantly extended the duration of mitosis in one-cell *C. elegans* embryos: the NEBD-anaphase interval was extended by 61% (from 188 ± 2.0 sec in control embryos to 303 ± 5.6 sec in BUB-1 depleted embryos). This extension was independent of checkpoint signaling as it was unaffected by co-depletion of MAD-2 and was observed in a checkpoint signaling-defective allele of *bub-1*. The promotion of anaphase onset by BUB-1 required kinetochore localization—mutants of BUB-3 and of the BUB-1/BUB-3 kinetochore receptor KNL-1 that perturbed kinetochore recruitment of BUB-1 delayed anaphase onset

to a similar extent as BUB-1 depletion. In addition, engineered mutant forms of KNL-1 that recruit variable amounts of BUB-1 to the kinetochore resulted in NEBD–anaphase onset durations that correlated with the magnitude of BUB-1 recruitment. To analyze the biochemical events underlying anaphase onset, we engineered a sensor for activation of separase, the protease that cleaves cohesin. Use of this sensor indicated that BUB-1 depletion delayed separase activation. Once activated, the kinetics of separase-mediated cleavage of the sensor was not affected by BUB-1 depletion, suggesting that the delay in separase activation is a consequence of delayed APC/C activation. Mutational analysis revealed that the BUB-1 kinase domain, but not its kinase activity, are important for promoting separase activation and anaphase onset. Notably, a mutant allele in the kinase domain that did not perturb chromosome alignment and rescued a *bub-1* null mutant delayed separase activation and anaphase onset. These results reveal an unexpected role for Bub1 in the promotion of anaphase onset, independently of its functions in checkpoint signaling and chromosome alignment, and suggest a new mechanism contributing to the switch-like transition from metaphase to anaphase.

P1789

Board Number: B313

N-terminal modification of Ndc80 can induce a spindle assembly checkpoint arrest in mouse oocytes.

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Correct microtubule-kinetochore (MT-KT) interactions are essential to achieve faithful chromosome segregation and prevent aneuploidy. The spindle assembly checkpoint (SAC) helps ensure the fidelity of this process, by coupling anaphase-onset with MT-KT attachment. A key component of the MT-KT interface is Ndc80 (Hec1 in humans), which is essential for maintaining kinetochore integrity and the stability of MT-KT interactions during mitosis. However, in mammalian oocytes, which are susceptible to aneuploidy, its function remains unclear. In this study, by expressing fluorescently tagged C- or N-terminally coupled Ndc80 protein in mouse oocytes, we examined the effect on chromosome dynamics and SAC activity. Both of the fused proteins showed specific kinetochore localization, however, majority of the N-terminal tagged Ndc80 (Ndc80-N) oocytes failed to complete meiosis I, and instead arrested at metaphase I (MI completion rates: 12.5% of Ndc80-N oocytes, n=80; vs. 67.5% of Ndc80-C oocytes, n=40; p<0.0001, Fisher's exact test), and this was associated with an increased percentage of incorrect MT-KT attachments (17.7% in Ndc80-N injected oocytes, n=355 kinetochores examined; vs. 7.6% in H₂O injected oocytes, n=158; p=0.0027, Fisher's exact test). Interestingly, live cell imaging revealed that these attachment errors had no significant effect on chromosome congression and bivalent tension establishment during meiosis I. Nevertheless, securin degradation in Ndc80-N expressing oocytes was prevented, suggesting inhibition of Anaphase-Promoting Complex/Cyclosome (APC/C) activity. Consistent with this, an increase in kinetochore Mad2 localization was identified (357 ± 14.9 arbitrary intensity in Ndc80-N injected oocytes, n=280 kinetochore examined; vs. 252 ± 12.4 in H₂O injected

oocytes, $n=280$; $p < 0.0001$, Mann-Whitney test). Furthermore, polar body extrusion (PBE) and securin degradation in these arrested oocytes was efficiently rescued by inhibiting either Aurora or Mps1 kinase, both of which are key SAC components (PBE rate: 20.9% in DMSO control group, $n=67$ oocytes examined; vs. 69.6% with Aurora inhibition, $n=46$; vs. 90.2% with Mps1 inhibition, $n=51$). Therefore, despite the fact that mammalian oocyte meiosis I is unique in having sister kinetochores that co-segregate in pairs, the data that modification of the N-terminal Ndc80 causes MT-KT attachment errors and a persistent SAC activation in mouse oocytes suggested that Ndc80 may serve a meiotic function that parallels its mitotic one, and emphasized the essential role of its N-terminal region in which interaction with microtubules occurs.

P1790

Board Number: B314

Defective post-transcriptional regulation of Mad2 by miR-493 induces mitotic anomalies in cells.

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Micro-RNAs (miRNAs) are highly conserved small non-coding RNA molecules (~21 nucleotides in length) implicated in the control of diverse biological processes such as cell differentiation, proliferation and growth. Altered miRNA expression is a contributing factor in cell transforming processes that can trigger cancer initiation or promote tumor growth and metastasis. A miRNA regulates gene expression by binding to complementary sequence in its target messenger RNA (mRNA), which is then directed for degradation or translational inhibition. Since mitotic fidelity is essential for maintenance of genomic balance and normal cell growth, we were interested to get insights on how miRNAs participate in regulation of mitosis. To this end, we designed and executed a high-throughput screen to identify miRNAs that abrogate normal Spindle Assembly Checkpoint (SAC) signaling when overexpressed. Here we report a novel mitosis perturbing miRNA, miR-493, which binds to the 3' untranslated region (3' UTR) of Mad2 mRNA and thereby controls the Mad2 protein levels in cells. Overexpression of miR-493 led to a significant suppression of Mad2 mRNA and protein levels, and reduced Mad2 accumulation to the unattached kinetochores. As a consequence, the cells underwent a forced mitotic exit and antagonized microtubule drug effects. Moreover, cells with excess miR-493 exhibited a significant increase in the frequency of aneuploidy, apoptosis and cellular senescence when compared to controls. The specificity of miR-493 action on Mad2 was confirmed using a Mad2-target site blocker, which competes with miR-493 for binding to Mad2 mRNA. The Mad2-target site blocker restored Mad2 protein levels, reactivated SAC, and prevented aneuploidy and cellular senescence caused by the excess miR-493. Our results reveal a new miRNA-mediated control mechanism of mitosis, deregulation of which can contribute to

genomic instability. Moreover, the findings can help to understand how Mad2 expression may be altered during malignant cell growth *in vivo*.

P1791

Board Number: B315

A genetic view of metazoan mitotic exit through Protein Phosphatase 2A.

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Mitotic progression is largely governed by kinases, phosphatases and ubiquitin ligases. Whereas high levels of CycB-Cdk1 kinase activity trigger mitotic entry, mitotic exit requires the inactivation of this kinase and the activation of one or several phosphatases at the onset of anaphase. It has recently been determined that PP2A-B55/Tws plays a major role in CycB-Cdk1 substrate dephosphorylation in mitotic exit in higher eukaryotes. The precise substrates that must be dephosphorylated by PP2A-B55/Tws to allow mitotic exit events including chromosome segregation are unknown.

Here, a genetic screen was used to identify genetic PP2A-B55/Twins collaborators. We exploited the fact that meiosis and early embryonic mitoses of *Drosophila* syncytial embryos are particularly sensitive to cell cycle perturbations. We conducted a second-site non-complementation, maternal effect lethal screen. Several PP2A-Twins collaborators for cell cycle progression such as CycB3, CENP-C, LamB and main components of the nuclear-cytoplasmic protein import-export machinery were identified. To identify potential genetic networks governing mitotic exit, hits were cross-screened using the same approach. Distinct genetic networks were identified, governed mainly by CycB3 and Polo, in addition to PP2A-Tws.

Taken together, our genetic results suggest that PP2A-B55/Twins regulates mitotic exit through several pathways. Identified PP2A-B55/Tws genetic interactors form distinct genetic networks that are likely to regulate several mitotic exit events such as chromosome segregation, chromosome decondensation, nuclear envelope reformation and spindle assembly checkpoint inactivation. Future work will be aimed at understanding how these genetic components regulate mitotic exit processes individually and within their proposed networks.

P1792**Board Number: B316****Characterization of a novel splice site mutation in *CASC5* identified in a large Pakistani primary microcephaly family.**S. Szczepanski^{1,2}, M. Hussain^{1,2}, S. Baig³, P. Nürnberg^{2,4,5}, A. Noegel^{1,2,4,5};

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Autosomal recessive primary microcephaly (MCPH) is a rare neurodevelopmental disorder characterized by the reduced size of the cerebral cortex at birth accompanied by intellectual disability whereas the architecture of the brain does not show any abnormalities. MCPH genes code for proteins implicated in cell division and cell cycle regulation thereby being important components controlling brain size as a consequence of a reduced pool of neurons. To date 13 causative genes, named *MCPH1*, *WDR62*, *CDK5RAP2*, *CASC5*, *ASPM*, *CENPJ*, *STIL*, *CEP135*, *CEP152*, *ZNF335*, *PHC1*, *CDK6* and *SASS6* have been described for this disease.

We ascertained a primary microcephaly family from a remote region of Pakistan. Combination of homozygosity mapping and whole-exome sequencing identified a novel splice site mutation (c.6673-19T>A) in *CASC5*, a gene encoding a protein important for kinetochore formation and proper chromosome segregation during mitosis. Post-hoc Sanger sequencing of patient cDNA revealed the skipping of exon 25 of *CASC5* causing a frameshift and introducing a premature stop codon (p.Met2225Ilefs*7) which ultimately resulted in a C-terminally truncated protein. Furthermore, we found a down-regulation of *CASC5* mutant mRNA as measured by quantitative RT-PCR.

Upon analyzing the patient primary fibroblasts we observed a defective nuclear structure as the major phenotype. Additionally, micronuclei were found in these cells. Immunofluorescence analyses of the patient fibroblasts revealed a mislocalization of *CASC5* in dividing cells during metaphase as compared to wild type. Since the nuclear abnormalities are considered as a hallmark of genetic instability, an altered DNA damage response was studied. Upon UV-DNA damage, a higher percentage of H2AX foci were detected in the mutant fibroblasts as compared to wild type. Comparable results were obtained in western blot analysis.

Taken together these results show not only a second novel pathogenic mutation in *CASC5* but also underscore the function of the gene in proper kinetochore formation. Moreover, we demonstrate the mutation's consequence on the chromosome integrity and therefore indicating a distinct role in genomic maintenance and stability of the cell.

P1793**Board Number: B317****Chromosomally unstable cancer cells develop adaptive resistance to targeted therapy.**B. Orr^{1,2}, L. Talje³, B.H. Kwok³, D.A. Compton^{1,2};¹Department of Biochemistry, Geisel School of Medicine at Dartmouth College, Hanover, NH, ²Norris Cotton Cancer Center, Lebanon, NH, ³Institute for Research in Immunology and Cancer, University of Montreal, Montréal, QC

Aneuploidy is a hallmark of solid tumors and many aneuploid tumor cells display high rates of chromosome mis-segregation in a phenomenon termed chromosomal instability (CIN). This property distinguishes cancer cells from normal cells providing an opportunity for cancer-specific targeted therapy. CIN is most commonly caused by the persistence of errors in kinetochore-microtubule (kMT) attachments. Moreover, it has been shown that many cancer cells with CIN have hyperstable kMT attachments and that strategically destabilizing kMT attachments by overexpressing the kinesin-13 proteins Kif2b and MCAK suppresses CIN in cancer cells. This establishes the principle that CIN can be suppressed by destabilizing kMTs, but the broader application of protein overexpression for this purpose has severe technical limitations. To overcome this technical constraint, we performed a high throughput screen to identify small molecules that specifically modulate the microtubule depolymerizing and/or ATPase activities of kinesin-13 proteins. This screen identified one compound (Cp57) that potentiates the microtubule depolymerizing activity of MCAK in vitro. Cp57 is cell permeable and specifically potentiates MCAK activity because it selectively destabilizes kMT attachments only during metaphase without affecting non-kMTs, and induces a reduction in MCAK phosphorylation at Serine 92 and 95. Furthermore, sensitivity to Cp57 decreases sharply in MCAK-depleted cells and increases in cells over-expressing MCAK. Importantly, short term treatment with Cp57 reduces the rate of lagging chromosomes in anaphase in CIN cancer cells with no detectable effect on chromosome segregation in diploid cells. Surprisingly, the rate of lagging chromosomes in anaphase and the phosphorylation of MCAK at Serine 92 and 95 rebound in CIN cancer cells treated with Cp57 for >72h. Moreover, kMT attachments become hyperstable in prometaphase of mitosis following treatment for >72h. Thus, the CIN status rapidly returns to the cancer cells. These data demonstrate that cancer cells with CIN rapidly acquire adaptive resistance to targeted therapy.

P1794**Board Number: B318****VTT-006, a novel anti-mitotic compound, binds to the Ndc80 complex and suppresses cancer cell growth in vitro.**

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Hec1 (Highly expressed in cancer 1), a member of the Ndc80 complex, resides in the outer kinetochore plate where it works to facilitate proper kinetochore-microtubule interactions. Hec1 is overexpressed in various cancers and expression shows correlation with high tumour grade and poor patient prognosis. Based on previous RNAi studies, chemical inhibition of Hec1 is anticipated to hyperactivate the spindle assembly checkpoint (SAC) and suppress cell proliferation which may possess therapeutic value. In this study, we designed and executed a high-throughput screen to identify novel low molecular weight (LMW) compounds that target Hec1 calponin homology domain (CHD), which is needed for normal microtubule attachments. First, 4 million compounds were virtually fitted against the CHD to discover compounds that potentially interfere with Hec1-microtubule interaction. This approach led to the identification of VTT-006 that showed binding to recombinant Ndc80 complex and modulated Hec1 association with microtubules in vitro. In cells, VTT-006 treatment resulted in chromosome congression defects, accumulation of Aurora B to centromeres, and loss of interkinetochore tension that triggered a SAC-dependent mitotic arrest followed by cell death. Furthermore, VTT-006 suppressed the growth of several cancer cell lines and enhanced the sensitivity of HeLa cells to taxol treatment. Our findings propose that VTT-006 is a potential anti-mitotic compound that disrupts normal mitotic progression by modulating kinetochore-microtubule interactions.

P1795**Board Number: B319****Abrupt sister chromatid splitting in anaphase without obligatory positive feedback.**J. Kamenz^{1,2}, T. Mihaljev³, S. Legewie³, S. Hauf^{1,4};¹Friedrich Miescher Laboratory, Max Planck Society, Tuebingen, Germany, ²Dept. of Biol. Sciences, Virginia Tech, Blacksburg, VA, ³Institute of Molecular Biology (IMB), Mainz, Germany, ⁴Dept. of Biol. Sciences and VBI, Virginia Tech, Blacksburg, VA

Major transitions from one cell cycle phase to the next are implemented with positive feedback loops in their regulatory pathways, which ensures that they occur quickly and are irreversible. The transition from metaphase to anaphase is visually one of the most dramatic transitions in the cell cycle. Since chromosomes split abruptly and synchronously, it is natural to assume that this transition involves positive feedback, and some evidence has been found in budding yeast. We now show that chromosome splitting can be slowed down and made more asynchronous by several different perturbations in fission yeast. We find a tendential order in the time when different chromosomes separate, which is aggravated when slowing down separation. Because affecting different steps in the segregation pathway all lead to a less abrupt separation, we conclude that feedback happens, if at all, far upstream, but not at the level of the direct regulators. Hence, sister chromatid separation, being already irreversible by nature, may be one of the few major cell cycle transitions that can operate without positive feedback.

P1796**Board Number: B320****Overexpression of Kif2b Acts Synergistically with K-Rras G12D Mutation to Accelerate Lung Tumor Progression in Mice.**C.D. Laucius^{1,2}, D.A. Compton^{1,3};¹Department of Biochemistry, Geisel School of Medicine at Dartmouth College, Hanover, NH, ²Norris Cotton Cancer Center, Hanover, NH, ³Norris Cotton Cancer Center, Lebanon, NH

Chromosomal instability (CIN) – a high rate of whole chromosome loss or gain - is a hallmark of many aneuploid solid tumors. CIN is frequently linked with drug resistance and poor patient prognosis. The persistence of mal-oriented attachment of spindle microtubules to kinetochores during mitosis is the most common cause of CIN. Correction of these mal-orientations relies on detachment of microtubules from kinetochores, a process stimulated by the microtubule-depolymerizing Kinesin 13 family motor proteins Kif2b and MCAK. We have previously shown that Kif2b overexpression is sufficient to suppress CIN in multiple human cancer cell lines grown in cell culture. Therefore, we hypothesized that Kif2b overexpression would suppress CIN in aneuploid solid tumors and would offer insight into the role of CIN in tumor initiation and/or progression. To test this, we constructed a Kif2b conditional

overexpression transgenic mouse (Kif2b COE). The transgene, containing a floxed translational stop sequence, was targeted to the endogenous mouse Rosa26 locus. Progeny of Kif2b COE mice crossed with transgenic CMV-Cre mice show robust Kif2b expression in all tissues. There is no change in the incidence of tumors in mice constitutively overexpressing Kif2b in an oncogenic K-Ras (G12D) lung cancer model. However, Kif2b expression enhanced tumor growth as the mean surface area (mm²) of pulmonary nodules increases from 0.27 to 0.43. Accordingly proliferating Ki-67 positive cells significantly increase upon Kif2b overexpression as compared to control animals. Unexpectedly, Kif2b overexpression increased the frequency of aneuploid cells from 3.8% to 6.3% ($p < 0.05$) in lung tumors driven by mutant K-Ras as judged by FISH for chromosome 2. Thus, there are distinct consequences of Kif2b overexpression in cultured cells relative to animals tissues, and we demonstrate that Kif2b overexpression drives tumor progression in the context of a mutant K-Ras G12D lung cancer model.

P1797

Board Number: B321

Role of phosphatases in chromosome segregation.

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Chromosome instability (CIN) is a hallmark of cancers. How chromosomes attach to microtubules is not fully understood and hence our goal is to study proteins that are essential for the early event of chromosome-microtubules (KT-MT) capture. Our studies confirm that the phosphatase PP2A recruited by BubR1 plays an important role in chromosome congression. Here we present data to show how the phosphatase controls KT-MT attachments.

P1798

Board Number: B322

Resolving the structure of the kinetochore: the story unfolds.

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The kinetochore is a highly complex proteinaceous structure located at the primary constriction of mitotic chromosomes. There, it performs an essential role in accurate chromosome segregation. More recently, interest has been directed towards the Constitutive Centromere Associated Network (CCAN) components, as they participate in the formation of a scaffold involved in kinetochore assembly. It is therefore important to fully understand their role, and their distribution, at the kinetochore. Although many kinetochore proteins have already been identified, it is still unclear how centromeric chromatin folds to form the structure of the inner kinetochore. This is an interesting yet still open field of study,

where the literature reports are still quite divided. In our study we take advantage of the high homologous recombination efficiency in DT40 B-lymphoma chicken cell lines, allowing us to obtain conditional knockouts and deletion cell lines of several CENPs subsequently engineered to stably express GFP:CENP-A. In the parental cell line the unfolding properties of the centromeric region were investigated by using TEEN buffer. Using fluorescence microscopy we were able to measure the length of many unfolded centromeric chromatin fibres, based on the signal of GFP:CENP-A. A multi-peak analysis revealed the presence of discrete populations of fibres, recognised as peaks, in both interphase and mitotic samples. Compared with interphase, mitotic centromeres showed a greater level of compaction. Next, mutants for CCAN components blocked in mitosis, were subjected to centromere chromatin unfolding. Results revealed that mitotic kinetochores depleted of CENP-C and CENP-S behaved similarly to the parental interphase samples, suggesting a role of those proteins in maintaining kinetochore structure. In contrast, CENP-O, CENP-H and CENP-I depletion in mitosis did not seem to weaken the structure of the kinetochore. Additionally, we tested a hypothesis suggested by the multi-peak analyses that chromatin layers exist in the inner kinetochore. Our data, when combined with published EM and crystallography measurements of centromere/kinetochore components, allowed us to assemble a robust and mathematically viable model that supports a boustrophedon organisation of the kinetochore chromatin fibre. This work represents an advance in our understanding of kinetochore structure in vertebrates.

P1799

Board Number: B323

Visualizing dynamic kinetochore structure using super-resolution microscopy.

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During mitosis, the functions of kinetochores change from interacting with the lateral surface of microtubules and activating the spindle assembly checkpoint (SAC) to capturing microtubule ends and silencing the SAC. The molecular mechanism that couples this functional change to microtubule attachment status remains unclear. Applying 3-D super-resolution imaging to *Xenopus* egg extracts, here we reveal that the kinetochore is spatially and functionally segmented into a stable core module supporting end-on attachment and an expandable module responsible for lateral attachment and SAC signaling. Unexpectedly, the inner kinetochore component CENP-C is an integral component of the expandable module, whose assembly also depends on outer kinetochore proteins (Bub1, BubR1) and multiple protein kinases (Aurora B, Haspin, Plx1, Mps1) and is suppressed by protein phosphatase 1. We propose that the expandable module consists of a phosphorylation-dependent copolymer that spatially segregates kinetochore functions to help couple end-on attachment and SAC silencing.

P1800**Board Number: B324****Oncogenic RAS/MAPK signaling hyper-activates Aurora B kinase and weakens kinetochore-MT attachments.**J.A. Herman¹, P.J. Paddison², J.G. DeLuca¹;¹Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO, ²Human Biology Division, Fred Hutchinson Cancer Research Center, Seattle, WA

For over a century it has been appreciated that many cancer cells exhibit a high degree of aneuploidy, which results from the incorrect segregation of whole chromosomes during mitosis. Recent evidence has also demonstrated that the chromosome segregation errors leading to aneuploidy commonly arise from defects in kinetochore-microtubule attachment regulation. Thus, successful chromosome segregation requires that kinetochores not only link the forces from dynamic spindle microtubules to chromosomes, but also precisely regulate the attachment strength to these microtubules. For example, in early mitosis, kinetochores prevent the stable binding to ends of microtubules emanating from the incorrect spindle pole to prevent the accumulation of attachment errors. Such error prevention is accomplished primarily through Aurora B kinase (ABK) dependent phosphorylation of the kinetochore factors responsible for binding microtubules and generating stable kinetochore-microtubule attachments. Despite a growing understanding of how cells become aneuploid and the mechanisms cells use to prevent this condition, the mechanism by which cancer cells become prone to errors in kinetochore-microtubule attachment remains poorly understood. To investigate this issue, we transformed primary RPE cells with various oncogenic stresses. In doing so, we found that oncogenic RAS/MAPK signaling compromises kinetochore-microtubule attachments through a phosphorylation cascade of kinetochore targets. Oncogenic RAS/MAPK signaling aberrantly hyper-activates the ABK error correction system and destabilizes kinetochore-microtubule attachments. This decreased attachment stability prolongs mitosis and increases chromosome segregation errors, and both defects are largely rescued by inhibition of RAS/MAPK signaling. This amplification of ABK activity also results in an increased dependence on recruitment of the ABK-counteracting phosphatase PP2A to kinetochores. In order to form stable kinetochore-microtubule attachments, RAS/MAPK-transformed cells require the BubR1-KARD domain, which contributes to PP2A kinetochore recruitment, while non-transformed cells do not. Together, these findings demonstrate that in at least a subset of cancers, kinetochore-microtubule attachments become weakened, and suggest that this defect may be exploited to generate new, highly specific therapeutics.

Spindle Assembly 3**P1801****Board Number: B325****Fission yeast Kinesin-8 controls chromosome alignment by containing sister chromosome oscillations and providing a length dependent pulling force.**

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The formation of a metaphase plate before chromosome segregation is a key step during mitosis in many species to maintain genetic stability. During this process, the motor protein Kinesin-8 is required to align chromosomes at the spindle center. In vitro evidences suggest that Kinesin-8 acts on microtubule depolymerization in a length-dependent manner but the mechanism by which this alignment occurs in vivo remains to be clarified.

Through a combination of 3D and high-speed video microscopy, we analyzed the effect of Klp5, the fission yeast Kinesin-8 homolog, on chromosome movement and positioning during mitosis. We show that Kinesin-8 accumulates at the plus-end of microtubules in mitosis and provides positional information by containing sister chromosome oscillations and restricting them at the spindle midzone during the entire metaphase. To test the possible mechanism at the origin of this centering defect, we used a simple force-balance model of the mitotic spindle. We demonstrate that a length-dependent pulling force exerted at the sites of attachment is necessary and sufficient to mimic the effect of Kinesin-8 on chromosome centering and to prevent the appearance of lagging chromosomes.

Altogether, these data bring new insight into how the local action of a single motor protein at the kinetochore can be integrated at the scale of the entire spindle to prevent aneuploidy.

P1802

Board Number: B326

Phosphorylation-dependent Regulation of the Checkpoint Kinase Kin4.

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Budding yeast cells are inherently polarized and asymmetrically dividing. Positioning of the mitotic spindle through the mother to daughter cell polarity axis is crucial for accurate chromosome segregation. The spindle position checkpoint (SPOC) is responsible for monitoring spindle orientation and delaying cell cycle progression in case of spindle misalignment. SPOC dependent cell cycle arrest relies on a bi-partite GTPase-activating protein (GAP) complex, composed of Bub2 and Bfa1, which inhibits the activation of the mitotic exit network (MEN, a GTPase driven signalling cascade essential for mitotic exit and cytokinesis). The function of Bub2-Bfa1 GAP complex is modulated by the protein kinase Kin4, which phosphorylates Bfa1 thereby keeping the GAP in the active state.

Kin4 kinase is a phospho-protein regulated at multiple levels. The phosphorylation status of Kin4 determines its activity and localization. Kin4 associates with the mother cell cortex during most phases of the cell cycle and localizes to the bud neck in late anaphase. Additionally, it binds to the mother spindle pole body (SPB, functional equivalent of centrosome in yeast cells) for a short period during

mitosis. However, after SPOC activation, Kin4 SPB localization becomes prominent on both SPBs. Kin4 cortex and SPB localizations and catalytic activity are phosphorylation dependent and regulated by protein phosphatase 2A (PP2A) subunit Rts1 and the kinase Elm1, respectively. Several evidences indicate the involvement of other elements to the Kin4 phospho-regulation.

In this study, we aimed to identify and characterize novel Kin4 regulators. For this, we performed a yeast kinome library screening to search for protein kinases involved in Kin4 phospho-regulation. In addition to the already known SPOC player Elm1, we identified Cdc28 as a putative kinase involved in Kin4 phosphorylation. Using genomic and cell biology approaches, we show that Cdc28 phosphorylates Kin4 *in vitro*. Furthermore, Kin4 phospho-deficient mutant at putative Cdc28 phosphorylation sites do not show *in vivo* catalytic activity and failed to engage SPOC. We propose that Cdc28 dependent phosphorylation of Kin4 is required for Kin4's role in SPOC.

P1803

Board Number: B327

Csi1p recruits alp7p/TACC to the spindle pole bodies for bipolar spindle formation.

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Accurate chromosome segregation requires timely bipolar spindle formation during mitosis. The transforming acidic coiled-coil (TACC) family proteins and the ch-TOG family proteins are key players in bipolar spindle formation. They form a complex to stabilize spindle microtubules, mainly dependent of their localization to the centrosome (the spindle pole body/SPB in yeast). The molecular mechanism underlying the targeting of the TACC-ch-TOG complex to the centrosome remains unclear. Here, we show that the fission yeast *Schizosaccharomyces pombe* TACC ortholog alp7p is recruited to the SPB by csi1p. The csi1p interacting region lies within the conserved TACC domain of alp7p while the carboxyl-terminal domain of csi1p is responsible for interacting with alp7p. Compromised interaction between csi1p and alp7p impairs the localization of alp7p to the SPB during mitosis, thus delaying bipolar spindle formation and leading to anaphase B lagging chromosomes. Hence, our study establishes that csi1p serves as a linking molecule tethering spindle stabilizing factors to the SPB for promoting bipolar spindle assembly.

P1804**Board Number: B328****Galectin-3 as a New Dynamic Regulator of the Spindle Pole Stability.**J. Magescas¹, L. Sengmanivong², F. Poirier³, D. Delacour¹;¹Institut Jacques Monod - CNRS, Paris, France, ²Nikon Imaging Center @ Institut Curie - CNRS, Paris, France, ³Institut Jacques Monod CNRS, Paris, France

Mitosis is a highly regulated cellular process, where many internal rearrangements occur, notably for the microtubule network. Through cell division, microtubules are rearranged from a centrosome-centered array to antiparallel arrays, namely mitotic spindles, at both cell poles. Spindle microtubules connect centrosomes to kinetochores, and motor proteins and organizing/stabilizing microtubules proteins regulate / ensure minus-end microtubule dynamics there. Astral microtubules link spindles to the cell cortex, allowing control of cell division positioning and orientation. These different steps ensure the correct segregation of the sister chromatid in both daughter cells, and any defects in microtubule organization will lead to chromosomal instability and generate aneuploidy. Recently a new kind of post-translational modification, the O-linked N-acetylglucosamine addition, was reported to regulate the spindle pole assembly. However no potential molecular mechanism has been yet identified at that level. One particular protein is interesting in this regard, Galectin-3, a lectin mainly expressed in epithelial cells. Its expression has been extensively correlated with cancer progression. At the cellular level, it is described as a multifunctional protein both intra- and extracellular. Most recently, our lab described Galectin-3 as a new key player in centrosome biology. During ciliogenesis, we reported that Galectin-3 transiently associates with basal bodies in kidney cells as well as in multiciliated trachea cells. There, Galectin-3 has been shown to be important for the recruitment of γ -tubulin and thus the anchorage of microtubules to the basal body MTOC (under revision) We investigated the potential participation of Galectin-3 in mitosis and spindle dynamics in vitro in different human epithelial cell lines. We found that Galectin-3 depletion generates a large panel of mitotic defects. At the spindle pole, Galectin-3 is located at the minus-end microtubules, and interacts with NuMA, a well-known microtubule array organizer in a glycosylation-dependent manner. Altogether, our data demonstrate that Galectin-3 behaves as an oligomerization factor for NuMA, and actively takes part in the cohesion of the spindle microtubule arrays.

P1805**Board Number: B329****Calmodulin and Abnormal Spindle Cooperate to Maintain Mitotic/Meiotic Spindle Integrity in *D. melanogaster*.**T. Schoborg¹, R.X. Guillen², N.M. Rusan¹;¹Cell Biology and Physiology Center, NHLBI, NIH, Bethesda, MD, ²National Heart Lung, and Blood Institute, NIH, Bethesda, MD

Autosomal Recessive Primary Microcephaly (MCPH) is a neurodevelopmental disorder characterized by reduced brain volume and head size, leading to mild mental retardation. Mutations in at least seven different genes have been linked to this disorder, most of which are involved in mitotic spindle function. The most frequent lesions map to the Abnormal Spindle-Like, Microcephaly Associated (ASPM) locus, the vertebrate ortholog of the Abnormal Spindle (Asp) gene from *Drosophila melanogaster*. Although Asp appears to be required to focus microtubule (MT) minus ends at spindle poles during mitosis, its molecular function and etiology remain poorly understood. Here we provide evidence that Asp directly interacts with Calmodulin (CaM) and both associate with spindle microtubules during mitosis and meiosis. Asp is required for CaM localization to the spindle, an interaction that is mediated through at least five separate IQ domains present in the C-terminal region of Asp. In support of their functional dependence, we show they exhibit nearly identical and dynamic streaming behavior towards MT minus ends. This occurs during metaphase and is lost upon CaM inhibition. Reduction in Asp protein levels leads to detachment of MT minus ends from spindle poles, which can be phenocopied through CaM inhibition or loss of the N-terminus of Asp. Our results suggest that CaM and Asp cooperate to maintain spindle integrity, providing significant insight into the molecular mechanisms underlying MCPH.

P1806**Board Number: B330****The far C-terminus of MCAK regulates its conformation and spindle pole focusing.**H. Zong¹, S.K. Lamb², C. Moe¹, S.L. Shaw¹, C.E. Walczak³, S.C. Ems-McClung¹;¹Department of Biology, Indiana University, Bloomington, IN, ²School of Medicine, Vanderbilt University, Nashville, TN, ³Medical Sciences, Indiana University, Bloomington, IN

Accurate chromosome segregation relies on the proper assembly of the bipolar spindle, which is governed in part by microtubule (MT) associated proteins that regulate MT dynamics. MCAK (mitotic centromere-associated kinesin), a member of Kinesin-13 family, depolymerizes MTs from both ends and is critical for proper spindle assembly. Inhibition of MCAK causes excess MT polymer and impedes spindle assembly. Furthermore, the ability of MCAK to undergo intramolecular conformational changes throughout its catalytic cycle is critical for MT depolymerization activity. MCAK consists of an N-terminal kinetochore targeting domain, a class-specific neck, a kinesin-like catalytic domain and C-terminal

dimerization domain. We previously identified S196 phosphorylation within the neck as a major regulator of MCAK conformation, which in turn regulates MCAK MT depolymerization activity. We also found that the conformational regulation of MCAK is dependent on the C-terminus, but how the C-terminal domain is involved in this regulation was not known. Through deletion analysis and site-directed mutagenesis we identified a region in the far C-terminus of MCAK that is important for its conformation and has a role in focusing spindle poles. Mutation of two conserved glutamate residues in the far C-terminus (E715 and E716) to alanine (715AA) causes increased Förster resonance energy transfer (FRET) in solution and faster fluorescence lifetimes on MTs, suggesting a more 'closed' conformation. In depletion/add-back assays in egg extracts, MCAK containing the 715AA mutation (MCAK-715AA) rescued spindle assembly, but 44% of spindles had unfocused poles. Addition of excess MCAK-715AA to extracts caused increased free chromatin, suggesting that MCAK-715AA had higher MT depolymerization activity. This might be caused either by increased biochemical activity or by elevated targeting to sub-regions of the spindle. Time-lapse imaging of EB1 tracks in spindles suggests that MCAK-715AA preferentially disrupts the polar MT array that emanates from the spindle poles. Interestingly, an Aurora A phosphomimetic S719E mutation in MCAK also has a more 'closed conformation' and leads to an increase in unfocused spindle poles when added to extracts. These data support the model that mutation of the far C-terminus of MCAK affects Aurora A-mediated phosphorylation, which regulates both MCAK conformation and activity. We are currently probing the role of S719 phosphorylation in the formation of disrupted polar array in extracts using live imaging.

P1807

Board Number: B331

Human γ TuRC in the mitotic spindle is associated with microtubule minus ends and mediates their poleward sorting, in addition to its essential role as nucleator.

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During mitotic spindle assembly multi-subunit γ -tubulin complexes nucleate microtubules at centrosomes, around mitotic chromatin, and, by interaction with augmin, from pre-existing microtubules. Whereas in yeast and flies a γ -tubulin small complex (γ TuSC) composed of only three subunits, γ -tubulin, GCP2, and GCP3, is sufficient for mitotic spindle assembly, the requirements in human cells are less clear. We will present evidence that centrosome targeting, nucleation and spindle assembly require the entire human γ TuRC and that human γ TuSC is not sufficient. In addition, we will provide insight into the non-centrosomal roles of γ TuRC within spindles. Our data indicates that γ TuRC is associated with minus ends of non-centrosomal spindle microtubules. Recruitment of γ TuRC to spindles occurs preferentially at pole-distal regions, requires nucleation and/or interaction with minus ends, and is followed by sorting of minus end-bound γ TuRC towards the poles. Poleward movement of γ TuRC exceeds k-fiber flux, involves the motors dynein, HSET (also known as KIFC1; Kinesin-14 family member), and Eg5 (also known as KIF11; Kinesin-5 family member), and slows down in pole-proximal regions,

resulting in the accumulation of minus ends. Thus, in addition to nucleating microtubules, γ TuRC actively contributes to spindle architecture by organizing microtubule minus ends.

P1808

Board Number: B332

Cell Cycle-Dependent Regulation of the TPX2-Eg5 Interaction.

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Eg5 is a plus-end directed mitotic kinesin that generates outward force to establish spindle bipolarity. Using TIRF microscopy of cells expressing Eg5-EGFP, we previously observed that Eg5 punctae moved poleward during early mitosis and away from the pole in anaphase. In cells depleted of the microtubule-associated protein, TPX2, Eg5 motion was away from spindle poles; motion was blocked following inhibition of dynein. These data support a model in which TPX2 links Eg5 to dynein for poleward motion during early, but not late, mitosis. In vivo and proteomic studies show that human TPX2 is phosphorylated at a residue within the C-terminal region that interacts with Eg5 (S738), suggesting that phosphorylation could regulate the Eg5-TPX2 interaction. To test this, we performed knock-down/rescue experiments in which GFP-tagged, siRNA resistant TPX2 constructs and TPX2 siRNA were co-nucleofected into LLC-Pk1 cells and spindle morphology was quantified. The results show that the majority of cells expressing wild-type and phosphomimetic (S738D) TPX2 constructs had bipolar spindles; however, cells expressing the non-phosphorylatable TPX2 mutant, S738A, showed significantly more multipolar spindles. These results were also confirmed in live cells expressing mCherry- α -tubulin. Because the Eg5-TPX2 interaction is necessary for kinetochore microtubule stability we expressed wild-type or mutant TPX2 and performed a cold stability assay. Preliminary data show that cells expressing TPX2-S738D had more stable K-fibers.

To further explore the interaction of Eg5 and TPX2, we performed in vitro, single molecule, assays using Eg5-EGFP from cytoplasmic extracts of LLC-Pk1 cells. The results show that Eg5 velocity was reduced by TPX2 and to a lesser extent by truncated TPX2 lacking the domain that interacts with Eg5. To determine if the phosphorylation state of TPX2 contributes to inhibition, we measured the velocity of Eg5 in the presence of either S738D, or S738A TPX2. We found that S738D was a more effective inhibitor of Eg5 motility than either wild-type or S738A TPX2, suggesting that TPX2 phosphorylated at S738 is better able to interact with Eg5 than either wild-type or S738A TPX2. Together our results show that the regulation of the essential mitotic motor, Eg5, by TPX2 is modified by phosphorylation at site S738.

P1809**Board Number: B333****Structural maintenance of chromosome (SMC) proteins link microtubule stability to genome integrity.**G. Laflamme¹, T. Tremblay-Boudreault¹, M. Roy¹, P. Andersen¹, E. Bonneil¹, K. Atchia¹, P. Thibault¹, D. D'Amours¹, B. Kwok¹;¹Institute for Research in Immunology and Cancer, University of Montreal, Montreal, QC

Members of the structural maintenance of chromosome (SMC) protein family are parts of the multi-protein complexes that are key organizers of chromosome architecture and are essential for genome integrity. The cohesin (Smc1-3) and condensin (Smc2-4) complexes are known to play critical role in sister chromatid cohesion and chromosome condensation, respectively. The relatively less understood Smc5-6 complex has been implicated in DNA repair, DNA replication and telomere maintenance. In general, SMC proteins act by binding to chromatin and connecting distinct parts of chromosomes together. However, their potential roles in providing direct connections between chromatin and the mitotic spindle has not been explored. Here, we present biochemical and genetic evidence to demonstrate that yeast SMC proteins bind directly to microtubules and can provide a functional link between microtubules and DNA. We mapped the microtubule-binding region of Smc5 (a component of the Smc5-6 complex) using a chemical-based approach and generated a mutant with impaired microtubule binding activity. This mutant is viable in yeast, but exhibited a cold-specific conditional lethality associated with mitotic arrest, aberrant spindle structures and chromosome segregation defects. In an in vitro reconstitution assay, this Smc5 mutant also showed a compromised ability to protect microtubules from cold-induced depolymerization. Collectively, these findings demonstrate that SMC proteins can bind and stabilize microtubules, and that SMC-microtubule interactions are essential to establish a robust system to maintain genome integrity.

P1810**Board Number: B334****The role of the actin-binding protein MISP in mitotic spindle orientation.**M. Zhu¹, Y. Schlosser¹, I.M. Hoffmann¹;¹Cell Cycle Control and Carcinogenesis, F045, German Cancer Research Center, DKFZ, Heidelberg, Germany

For accurate placement of the cleavage plane, the mitotic spindle needs to be precisely oriented. Spindle positioning is mediated through a cortical machinery by capturing astral microtubules thereby generating pushing/pulling forces at the cell cortex. These cortical cues are spatially defined by retraction fibers modulating the positioning of actin regulators and therefore force generation. Moreover, astral MTs are engaged with these cortical structures through so-called +TIPs including adenomatous polyposis coli (APC), CLASPs and the dynein/dynactin complex, which have been shown to regulate spindle orientation and positioning. The cortically localized dynein/dynactin complex is believed

to provide pulling forces on astral MTs and is recruited by heterotrimeric G proteins/LGN/NuMA during spindle positioning in *C. elegans* embryos, *Drosophila* and human cells. However, the detailed mechanisms underlying the correct positioning and orientation of the mitotic spindle are still not fully understood and further investigation is needed to identify potential missing components involved in this process.

We have recently identified the actin-binding protein MISP as mitotic phosphoprotein and a substrate of Plk1 and Cdk1 that is required for correct positioning of the mitotic spindle. Depletion of MISP leads to a Plk1-dependent block in mitosis at the metaphase to anaphase transition with fragmented centrosomes, scattered chromosomes and misoriented, rocking spindles. Loss of MISP induces mitotic defects including spindle misorientation that is accompanied by shortened astral microtubules. Furthermore, we find that MISP forms a complex with and regulates the cortical distribution of the +TIP binding protein p150glued, a subunit of the dynein/dynactin complex. We further investigated the function of MISP in spindle orientation by identifying interacting proteins of MISP in mitosis to better understand how MISP establishes a link between astral microtubules and the cell cortex to ensure proper mitotic spindle positioning.

P1811

Board Number: B335

Elucidating the role of the RNA binding exon junction complex in mitosis.

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Mitosis ensures accurate chromosome segregation and requires many key regulators to organize the mitotic spindle and control cell division. There is increasing evidence implicating RNA binding proteins in the spatial and temporal coordination of RNAs throughout the cell cycle. What remains underexplored is how these proteins regulate mitosis. The exon junction complex (EJC) is an RNA binding complex known to regulate RNA localization, translation, and nonsense mediated decay. Intriguingly, our lab discovered that loss of one EJC component Magoh prolongs prometaphase in both cultured cells and the developing brain. In this study we follow up on this discovery by addressing the following questions: 1) Does Magoh regulate mitosis through EJC function, and 2) What is the cellular basis of these mitosis phenotypes? We discovered that knockdown of any of three core EJC components, Magoh, Rbm8a, and Eif4a3, results in loss of a bipolar spindle in HeLa cells. We are currently assessing whether formation of a complex is necessary for mitosis. Additionally, these proteins localize to the mitotic spindle. Further characterization of HeLa cells shows that after EJC knockdown the centrosomes fail to separate, but their maturation is intact. We tested spindle integrity by examining the microtubule subsets within a mitotic spindle. EJC knockdown spindles have phenotypically normal k-fibers with an intact connection to the centromere and an active spindle assembly checkpoint. Our future studies are aimed at determining how the EJC functions molecularly to regulate mitosis and if EJC localization impacts mitosis. In particular we are following up on the exciting hypothesis that EJC function may be influencing

the levels of candidate mitotic regulators. Understanding this will further elucidate the critical regulation of mitosis by RNA binding proteins.

P1812

Board Number: B336

Mitotic motor CENP-E cooperates with PRC1 in temporal control of central spindle assembly.

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Mitotic motor CENP-E plays key roles in chromosome congression and spindle checkpoint satisfaction. We have recently identified and characterized syntelin, a novel allosteric inhibitor selective for CENP-E (Ding et al., 2010. Cell Res. 20, 1386-1390). Cells treated with syntelin progress through interphase, enter mitosis normally with a bipolar spindle and lagging chromosomes around the poles. Syntelin is an allosteric inhibitor which tightens CENP-E-microtubule interaction by slowing inorganic phosphate release. To delineate the role of CENP-E in reorganization of interpolar microtubules into an organized central spindle, metaphase synchronized cells were exposed to syntelin and other mitotic motor inhibitors. Syntelin does not perturb inter-polar microtubule assembly but abrogates the anti-parallel microtubule bundle formation. Real-time image shows that CENP-E inhibited cells undergo central spindle splitting and exhibits chromosome instability phenotypes. Using bio-syntelin affinity matrix, we have isolated CENP-E complex containing PRC1. Interestingly, inhibition of CENP-E did not alter the interaction between CENP-E and PRC1 but perturbed temporal assembly of PRC1 to the midzone. Surprisingly, inhibition of CENP-E perturbs the temporal control of PRC1 dephosphorylation which led to a persistent phosphorylation of PRC1 and an inhibition of central spindle assembly. These findings reveal a previously uncharacterized role of CENP-E motor in temporal control of central spindle assembly. Currently, we are reconstituting the central spindle sliding assay in vitro and test how CENP-E-PRC1 interactions orchestrate this process.

P1813

Board Number: B337

Differential sensitivity of non-transformed vs. chromosomally-unstable cells to partial inhibition of dynein.

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We have recently demonstrated that poleward transport of chromosomes during anaphase in mammalian cells is enacted by two parallel yet distinctly different mechanisms. In the first mechanism,

poleward movement is coupled with the depolymerization of microtubules at the kinetochore. This mechanism, commonly referred to as 'Pacman' activity, is utilized by the majority of chromosomes in human cells. However, Pacman activity is suppressed on some chromosomes that are instead propelled by a dynein-mediated transport of the stable microtubule fiber attached to the kinetochore (Sikirzhytski et al., 2014, JCB, 206:231-243). The number of chromosomes segregating via dynein-mediated transport appears to vary in different cell types. This variability suggests that partial inhibition of dynein may selectively suppress growth of those cell types that rely more heavily on the dynein-mediated transport for chromosome segregation. To test this prediction we compare long-term effects of the cell-permeable dynein inhibitors Ciliobrevins on the growth of non-transformed vs. transformed chromosomally unstable (CIN) human cell lines. A general trend emerging from these comparisons is that chromosomally stable cells (e.g., RPE1) continue to progress through the cell cycle and divide normally when treated with concentrations of Ciliobrevin that block proliferation of chromosomally unstable transformed cells (e.g., U2-OS, HeLa). Partial inhibition of dynein in CIN cells results in prolongation of mitosis and a marked increase in the number of chromosome segregation errors as manifested by lagging chromosomes and formation of micronuclei. While the duration of mitosis increases in both non-transformed and CIN cell lines treated with Ciliobrevin, non-transformed cells progress through multiple cell cycles with normal timing. In contrast, progeny of CIN cells that undergo mitosis in the presence of Ciliobrevin tend to arrest in interphase and many subsequently die. Even those cells that ultimately re-enter mitosis are delayed in interphase. This pattern is consistent with the notion that partial inhibition of dynein increases the probability of chromosome missegregation and this increase becomes lethal in those cells that are already CIN. Differential sensitivity of non-transformed vs. CIN cell lines to dynein inhibition may open an opportunity to selective killing of cancer cells with low concentrations of anti-dynein inhibitors.

P1814

Board Number: B338

Extended prometaphase arrest induces centrosome fragmentation in cultured human cells.

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The spindle assembly checkpoint (SAC) serves to protect chromosomal ploidy by blocking mitotic progression until all sister chromatid pairs achieve bi-orientation, and SAC activation has been used for decades to synchronize cells in mitosis. However, SAC inhibition of the Anaphase Promoting Complex (APC) is leaky, and the low-level cyclin B degradation eventually results in mitotic slippage. Recent studies suggest that even a moderate delay in mitotic progression can have significant effects on the fates of the daughter cells, including partial caspase activation and irreversible, p53-dependent cell cycle arrest. Indeed, when G2-synchronized cultures of Retinal Pigmented Epithelial (RPE1) cells were arrested in prometaphase for defined periods of time, there was a linear increase in ectopic microtubule

organizing centers (MTOC) once cells were released from mitotic arrest. Ectopic MTOC formation occurred regardless of the method used to achieve mitotic arrest, with peak MTOC formation observed at 8 hours of mitotic arrest. Immunolocalization of pericentrin revealed that in contrast to cells released from G2 arrest alone, cells that experienced prolonged mitotic delay exhibited fragmented centrosomes, with foci either clustered at the pole or as a distinct organizing center. However, centrosome fragmentation could be partially suppressed if RPE1 cells were depleted of separase or treated during mitotic arrest with the APC inhibitor, TAME. Together, these results suggest that the leaky activity of APC during mitotic arrest may compromise centrosome integrity, either by separase-mediated centriole disengagement or by APC-mediated degradation of substrates required for spindle pole integrity.

P1815

Board Number: B339

Budding yeast YBR296C-A encodes a protein that can inhibit APC-Cdc20 in vitro and contains amino acid motifs conserved with human p31comet.

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The spindle checkpoint ensures accurate chromosome segregation until spindle microtubules attach to all kinetochores correctly during mitosis. p31comet is an antagonist of the spindle checkpoint effector Mad2, which can abolish the Mad2-dependent spindle checkpoint. A BLAST search of the budding yeast proteome using human p31comet yielded a putative protein encoded by an uncharacterized potential open reading frame *YBR296-A*. We have observed evidence for an mRNA and protein product produced by the putative ORF *YBR296-A*. Analysis of the protein product of *YBR296-A* demonstrates it can inhibit APC^{Cdc20} activity *in vitro*. Mutations in 5 conserved C-terminal amino acids, the 'TISLS' amino acid motif that is shared with human p31comet, weakened the proteins APC^{Cdc20} inhibitory activity. *In vivo* deletion of *YBR296-A* yielded a slight sensitivity to the microtubule poison benomyl. We have named the ORF Tiny Yeast Comet1 (*TYC1*), and are working to further characterize its function.

P1816

Board Number: B340

A peptide inhibitor of APC-Cdc20 based on the Cdc20 Mad2-binding motif can directly bind to the APC.

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In mitosis, the spindle checkpoint ensures genome stability by delaying the onset of anaphase until all kinetochores are properly attached to the mitotic spindle. Previous studies have shown that some tumor cells treated with anti-mitotic drugs resulted in cell slippage, while after Cdc20 was knocked down by siRNA, it led to robust mitotic arrest followed by cell death. Thus, APCCdc20 inhibitors may

have potential as anti-cancer agents. We had previously observed that a Mad2-binding motif called DQ36 could inhibit APC^{Cdc20} activity *in vitro*. Here, we report that the DQ36 peptide containing Mad2-binding motif sequence can bind directly to the APC *in vitro*. Apc9 contains two small amino acid motifs similar to sequences in Mad2. We created an *apc9Δ* strain to test whether the Apc9 subunit was required for DQ36 to interact with the APC. Our data show that DQ36 peptide bound to APC^{Apc9Δ} as same as wild type APC, indicating that the Apc9 subunit is not required for the APC-DQ36 interaction. We are currently exploring which APC subunit interacts with the DQ36 inhibitor by employing peptide cross-linking experiments.

P1817

Board Number: B341

Expression in vivo of peptide inhibitors of APC-Cdc20 reduces growth rate.

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The mitotic spindle checkpoint monitors proper chromosome segregation. Damage to the checkpoint can lead to aneuploidy, potentially contributing to tumorigenesis. Current anti-cancer strategies, such as microtubule poisons, focus on activation of the spindle checkpoint. Unfortunately, cancer cells may undergo mitotic slippage in the presence of an activated checkpoint, resulting in re-entry into the cell cycle. Evidence has emerged to inhibition of the E3 ubiquitin ligase Anaphase-Promoting Complex (APC) or its co-activator Cdc20 can prevent mitotic slippage. In this study, we created a 65 amino acid peptide, named PQ65, based on Cdc20 of *S. cerevisiae* including the 'KILR' motif. PQ65 disrupted APC^{Cdc20} activity *in vitro*. A similar inhibitory effect was observed when PQ65 was narrowed down to its C-terminal 36 amino acids, called DQ36. To validate the inhibitory activities of PQ65 and DQ36 *in vivo*, we constructed a peptide over-expressing system employing the *pGAL1-10* promoter where the protein products included a nuclear localization sequence encoded upstream of the peptide. Constructs were integrated into the yeast genome by homologous recombination. Yeast induced to express either PQ65 or DQ36 in the presence of galactose displayed reductions in growth rates relative to the control strain. In conclusion, two peptides, PQ65 and DQ36, can inhibit APC^{Cdc20} activity *in vitro* and regulate cell cycle timing by reducing the growth rate when expressed *in vivo*, supporting the potential development of these peptides as targeted APC^{Cdc20} inhibitors.

P1818

Board Number: B342

Quantitative Assessment of Chromosome Instability Promoted by Chemotherapeutic Agents.

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Most solid tumors are aneuploid, carrying an abnormal number of chromosomes, and they missegregate whole chromosomes in a phenomenon termed chromosome instability (CIN). CIN is associated with poor prognosis in many cancer types, and targeting of CIN is an attractive strategy for anti-cancer therapeutics. The mechanisms causing CIN and its contributions to tumor initiation and growth are not well defined, partly because there is no straightforward, quantitative assays for CIN in human cells. To address this problem, we have developed the first Human Artificial Chromosome (HAC)-based quantitative live-cell assay for mitotic chromosome segregation in mammalian cells, with which we can easily score the rates of CIN within one cell division under different experimental conditions.

We have constructed a HAC encoding copies of enhanced green fluorescent protein (eGFP) fused to the destruction box (DB) of anaphase promoting complex/cyclosome (APC/C) substrate hSecurin. This HAC also contains tet operator (tetO) arrays and sequences encoding the tetracycline repressor fused to monomeric cherry fluorescent protein (tetR-mCherry). We have produced human U2OS cells carrying this HAC, in which we monitor HAC segregation in two ways: First, APC/C degrades the DB-eGFP fusion expressed from the HAC at anaphase onset, and DB-eGFP re-accumulates in the daughter cells after G₁ phase, when APC/C becomes inactive. Daughter cells that do not obtain a copy of the HAC will thus be GFP negative in the subsequent interphase. Using this system, we can not only detect HAC mis-segregation within a single cell division but also extrapolate the rate of mis-segregation after a defined number of cell divisions from the proportion of GFP positive cells. Second, because tetR-mCherry binds to the tetO arrays, the HAC itself could be followed by live imaging. HAC-bearing U2OS cell lines show low inherent levels of CIN, but HAC mis-segregation is markedly increased by treatment with chemotherapeutic drugs such as Reversine, an inhibitor of Mps1, and microtubule agents Nocodazole and Taxol.

In summary, we have developed new assays to score CIN levels in human cells and have shown that CIN levels increase upon treatment with chemotherapeutic agents, which makes our assays ideal for chemical screens.

P1819

Board Number: B343

Sliding filament mechanism for anaphase B – evaluation of “slide-and-cluster” vs “slide-and-flux-or-elongate” models.

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Chromosome segregation during mitosis involves chromosome-to-pole motility (anaphase A) and spindle elongation (anaphase B). We propose that, in *Drosophila* embryos, anaphase B depends on a “slide-and-flux-or-elongate” mechanism in which persistent, outward, kinesin-5-driven interpolar (ip) microtubule (MT) sliding “engages” to push apart the spindle poles when poleward flux is turned off. Thus, in pre-anaphase B (i.e. metaphase and anaphase A) spindles, the outward sliding of ipMTs is balanced by the depolymerization of their minus ends at the poles producing poleward flux, while the

spindle maintains a constant, steady state length. Following cyclin B degradation, however, ipMT depolymerization ceases so the sliding ipMTs can push the poles apart as poleward flux is turned off (Brust-Mascher et al, 2004; Wang et al, 2013). The competing “slide-and-cluster” model (Burbank et al., 2007) proposes that MTs nucleated at the equator are slid outward by the actions of the bipolar kinesin-5 plus a minus end directed motor which then pulls MTs inward as they approach the poles and clusters MTs at the poles. Here, we quantitatively compare these two models using data from *Drosophila* syncytial embryos. In both models, kinesin-5 crosslinks both parallel and anti-parallel (AP) MTs with a 3-fold preference for AP pairs which it can also slide apart (Van Wildenberg et al., 2008). In the slide-and-cluster model, minus end-directed motors bind to each MT minus end and walk on an adjacent, parallel MT if possible, while in the slide-and-flux-or-elongate model, all MT minus ends reaching the pole are depolymerized by kinesin-13. In both models MT plus ends exhibit dynamic instability described by growth and shrinkage velocities, as well as rescue and catastrophe frequencies. We find that in both models, dynamic instability parameters need to be fine-tuned to maintain a steady length spindle with fast turnover dynamics. Both models reproduce the pre-anaphase B spindle steady state length and dynamics (as evaluated using *in-silico* FRAP) as well as the rate of anaphase B spindle elongation. However, only the slide-and-flux-or-elongate model reproduces the change in MT dynamics observed experimentally at anaphase B onset (Cheerambathur et al., 2007) and consequently only this model fits all the data for *Drosophila* syncytial embryo mitotic spindles. Thus our work highlights the importance of acquiring and quantitatively evaluating experimental data on different dynamic aspects of spindle behavior in order to evaluate competing models for mitosis.

P1820

Board Number: B344

Midzone organization by the microtubule crosslinker, Feo, is required for kinesin-5-driven anaphase B in *Drosophila* embryos.

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During mitosis, chromosomes are separated by chromosome-to-pole motility (anaphase A) and spindle elongation (anaphase B). In *Drosophila* embryos, we propose that anaphase B depends on a “slide-and-flux-or-elongate” mechanism in which kinesin-5 motors at the spindle midzone drive outward sliding of anti-parallel interpolar (ip) microtubules (MTs) to push apart the poles when poleward MT flux is turned off. Here we investigated the contribution of the midzone-associated non-motor MAP, Feo, which shares with kinesin-5 a significant preference for crosslinking MTs into anti-parallel versus parallel polarity patterns. While Feo homologues (Ase1p/PRC1) have been shown to enhance the midzone localization of the MT-MT crosslinking motors, kinesin-4, -5 and -6 in other systems, we find that Feo antagonizes the association of kinesin-5 with the anaphase B spindle midzone. The midzone association of kinesin-5 is enhanced following antibody-induced dissociation of Feo but kinesin-5 cannot substitute for Feo function because the resulting midzone is abnormally narrow and anaphase B is impaired. Thus,

Feo organizes antiparallel ipMTs of the midzone into a configuration that facilitates the kinesin-5-driven sliding filament mechanism underlying anaphase B spindle elongation

P1821

Board Number: B345

Myosin-10 and Wee1 assist mitotic spindle positioning in *Xenopus laevis* embryonic epithelia.

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Maintenance of epithelial organization is dependent on the final position and orientation of the mitotic spindle. In order to investigate the mechanism controlling spindle orientation, we have performed an analysis of mitotic spindle dynamics in the embryonic epithelium of *Xenopus laevis*. First, we assessed the rotation of mitotic spindles, revealing a phase of directed rotation followed by a phase of stable rotational oscillations. Second, we identified the actin and microtubule binding motor protein Myosin-10 as an important part of the system controlling these oscillations. Knockdown of Myosin-10 results in structural instability of the spindle as well as greatly reduced rotational movement. Disruption of Myosin-10 function through expression of its MyTH4-FERM cassette results in spindle instability, exaggerated spindle movement, and spindle mis-positioning. Finally, we identified an interaction between Myosin-10-MyTH4-FERM and the Cdk1 inhibitory kinase Wee1. Inhibition of Wee1 decreased duration of mitosis and produced erratic spindle movement. Furthermore, depletion of Wee1 is sufficient to rescue many of the phenotypes of Myosin-10 depletion. Thus, Myosin-10 and Wee1 are essential for the normal function of the mitotic spindle positioning mechanism.

P1822

Board Number: B346

WRAD complex participates in a SET domain-independent function of Mixed-lineage leukemia (MLL) protein to ensure proper chromosome segregation during mitosis.

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Histone 3 lysine 4 tri methylation is a mark of active gene transcription and this mark is deposited by a family of Su(var)3-9, Enhancer of Zeste, Trithorax (SET) domain containing proteins in mammals. Mixed-lineage leukemia (MLL) protein, a member of this family, is best characterized for its essential role in

Hox gene regulation during development and its chromosomal translocations associated with aggressive leukemias. Like their yeast counterpart Set1, MLL (and other SET family members), occur in COMPASS-like multi-subunit complex, which consists of four common structural components called WDR5, RbBP5, Ash2l and Dpy30 (WRAD). WRAD is essential for stability and histone methyl transferase (HMT) activity of MLL.

However, the functions of MLL in cellular processes like cell-cycle regulation are not well studied. Here we show that the MLL has a regulatory role during multiple phases of the cell cycle. RNAi mediated knockdown reveals that MLL regulates S phase progression and, proper chromosome segregation and cytokinesis during M phase. Based on reconstitution of knock-down cells with mutant MLL, we narrow the cell-cycle regulatory role to the C subunit of MLL. Our analysis reveals that the transcriptional activation domain and not the SET domain is important for the S phase function of MLL.

Surprisingly, disruption of MLL-WRAD interaction is sufficient to disrupt proper mitotic progression. These mitotic functions of WRAD are independent of SET-domain of MLL and, therefore, define a new role of WRAD in subset of MLL functions. Further characterization of loss-of-function of MLL complex exhibits delay in mitotic progression and prolonged prophase. Immunofluorescence staining and time-lapse microscopy reveals that MLL knockdown results in defects in chromosome congression, and DNA alignment, resulting in chromosome loss. Finally, we address the overlapping and unique roles of the different SET family members in the cell cycle.

P1823

Board Number: B347

Regulation of spindle length by microtubule-destabilizing enzymes.

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The transmission of a complete genome to each daughter cell requires the correct partitioning of chromosomes in every cell division. In eukaryotes this process is achieved by the spindle. Although the metaphase spindle appears static, it is actually a highly dynamic structure composed of short-lived microtubules (MTs). Once assembled, it can last for hours and its structure continuously rebuilds itself to maintain its size and shape. The steady state results from the competition of motor proteins, dynamic instability and poleward flux of MTs. MTs focusing into spindle poles has been initially thought to be a result of their nucleation from centrosomes, however in plant mitosis and meiosis of most of animal species, MTs grow from multiple sites around chromatin and self-organize into a bipolar spindle even in the absence of centrosomes. MTs undergo poleward flux, which is a result of coordinated MT sliding and pole-localized disassembly factors. The characteristic bipolar shape is generated by the action of two molecular motors: (i) plus-end directed kinesin-5 (Eg5) cross-links and slides MTs poleward, generating an outward force, and (ii) the minus-end directed dynein focuses MTs creating spindle poles. Dynein is required for the spindle pole formation, both in the presence and in the absence of centrosomes and its function depends strongly on its interaction with dynactin, which increase dynein's processivity in vivo

and in vitro. By inhibiting the interaction between dynein and dynactin we can trigger the dissociation of the complexes leading to disruption of spindle poles. Cell-free extracts prepared from unfertilized *Xenopus laevis* eggs have been used to study spindle assembly in vitro because spindles are not constrained in any fixed volumes, which allow investigating the assembly mechanism more in details. Here we study the difference between the mechanisms of two commonly used dynein inhibitors: dynamitin and CC1. Whereas the size of dynamitin-treated spindles is not altered, CC1-treated spindles elongate dramatically because of the displacement of MT destabilizing enzymes from spindle poles without affecting MT growth rate and poleward flux. These recent findings will be implemented into a computational model of MT nucleation and spindle assembly, which will help us to better understand the importance of pole integrity in spindle formation and maintenance.

Chromosome Organization

P1824

Board Number: B348

Multiple requirements of PLK1 during mouse oocyte maturation.

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Polo-like kinase 1 (PLK1) orchestrates multiple events of cell division. Although PLK1 function has been intensively studied in centriole-containing and rapidly cycling somatic cells, little is known about its function in the meiotic divisions of mammalian oocytes, which arrest for a long period of time in prophase before meiotic resumption and lack centrioles for spindle assembly. Here, using specific small molecule inhibition combined with live mouse oocyte imaging, we comprehensively characterize meiotic PLK1's functions. We show that PLK1 becomes activated at meiotic resumption on microtubule organizing centers (MTOCs) and later at kinetochores. PLK1 is required for efficient meiotic resumption by promoting nuclear envelope breakdown and chromosome condensation, partly independently of CDK1. PLK1 is also needed to recruit centrosomal proteins to acentriolar MTOCs to promote bipolar spindle formation, as well as for stable kinetochore-microtubule attachment. Consequently, PLK1 inhibition leads to metaphase I arrest with misaligned chromosomes activating the spindle assembly checkpoint (SAC). Unlike in mitosis, the metaphase I arrest is not bypassed by the inactivation of the SAC. We show that PLK1 is required for the full activation of the anaphase promoting complex/cyclosome (APC/C) by promoting the degradation of the APC/C inhibitor EMI1 and is therefore essential for entry into anaphase I. Moreover, our data suggest that PLK1 is required for proper chromosome segregation and the maintenance of chromosome condensation during the meiosis I-II

transition, independently of the APC/C. Thus, our results define the meiotic roles of PLK1 in oocytes and reveal interesting differential requirements of PLK1 between mitosis and oocyte meiosis in mammals.

P1825

Board Number: B349

A cell free assay to study chromatin decondensation at the end of mitosis.

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Chromatin undergoes fascinating structural and functional changes during the metazoan cell cycle. It massively condenses at the beginning of mitosis with a degree of compaction up to fiftyfold higher than in interphase. At the end of mitosis, mitotic chromosomes decondense to re-establish their interphase chromatin structure. This process is indispensable for reinitiating transcription and the perpetuation of genomic information, and is thus of central importance in the cellular life cycle. Despite its significance to basic research as well as its potential medical implications, postmitotic chromatin decondensation is only poorly understood. It has been well described cytologically, but we lack an understanding of the underlying molecular events. We are ignorant about the proteins that mediate chromatin decondensation, the distinct steps in this multi-step procedure and their regulation.

We have developed an *in vitro* assay, which recapitulates the process in the simplicity of a cell free reaction. Highly compacted chromatin isolated from metaphase arrested HeLa cells is incubated with *Xenopus laevis* egg extract. This induces faithful chromatin decondensation in a time dependent manner. At the end of a process the chromatin is surrounded by a nuclear envelope with functional nuclear pore complexes. We show that this process depends on ATP and GTP hydrolysis. Furthermore, using a fractionation approach we identified the ATPases pontin and reptin (RuvBL1 and RuvBL2, respectively) as chromatin decondensation factors. These proteins and their ATPase activity are essential for decondensation *in vitro*. The fact that chromatin decondensation also depends on GTP hydrolysis suggests that also a GTPase is involved in this process. The elucidation of this GTPase is still ongoing.

P1826**Board Number: B350****The DNA damage response during mitosis induces whole chromosome mis-segregation and reduces cellular viability.**S.F. Bakhoun¹, L.C. Kabeche², J. Murnane³, B.I. Zaki⁴, D.A. Compton^{5,6};

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The DNA damage response (DDR) prevents cells from entering mitosis in the presence of DNA breaks. However, cancer cells often encounter DNA damage during mitosis secondary to checkpoint slippage with persistence of pre-mitotic damage or due to de novo induction of DNA breaks by therapeutic agents such as ionizing radiation. Mitotic cells do not possess the capacity to repair DNA breaks. Yet, DNA damage during mitosis results in a partial DDR, whose consequences, at a time when the cell is chiefly preoccupied by the process of chromosome segregation, remains elusive.

Here we show that activation of the DDR during mitosis selectively stabilizes kinetochore-microtubule (k-MT) attachments to chromosomes through Aurora-A and Plk1 kinases, thereby increasing the frequency of lagging chromosomes during anaphase. These lagging chromosomes subsequently generate a preponderance of micronuclei that predispose chromosomes to subsequent catastrophic pulverization. Genetic depletion or pharmacologic inhibition of DDR proteins, ATM or Chk2, abolishes the effect of DNA damage on both k-MTs and chromosome segregation. Activation of the DDR in the absence of DNA damage is sufficient to induce chromosome segregation errors. Interestingly, inhibition of the DDR during mitosis, in chromosomally unstable cancer cells with pervasive DNA damage, suppresses inherent chromosome segregation defects.

We then examine the consequences of generating lagging chromosomes in response to mitotic DNA damage on cellular viability. We find that suppressing chromosome mis-segregation by reducing k-MT stability leads to reductions in downstream chromosomal defects and a significant increase in the viability of irradiated mitotic cells. Further, orthotopically transplanted human glioblastoma tumors in which chromosome mis-segregation rates have been reduced are rendered markedly more resistant to ionizing radiation, exhibiting diminished markers of cell death in response to treatment.

In summary, activation of the DDR during mitosis inappropriately stabilizes k-MTs creating a link between structural and numerical chromosomal instabilities. Our work identifies an unexpected adaptive pathway in response to DNA damage during mitosis that propagates genomic instability and genetic heterogeneity at the expense of cellular viability.

P1827**Board Number: B351****Paired movement of the kinetochores and dynamic microtubules can explain the observed capture times of the kinetochores during meiosis I.**G. Cojoc¹, A. Florescu², A. Krull³, N. Pavin⁴, F. Jülicher², I.M. Tolic^{5,6};

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Accurate cell division depends on proper segregation of chromosomes. This requires all chromosomes to be attached to the spindle microtubules (MTs) via the kinetochores (KCs). The mechanisms that facilitate the interaction between MTs and KCs have been debated for a long time. However, all previous models are based on in vitro or perturbed in vivo systems and a precise contribution of individual mechanisms is still unknown. In this study we analyze the kinetics of KC capture in an unperturbed system in vivo, in combination with a theoretical model. A model organism where the capturing mechanisms can be followed live with high spatial and temporal resolution is *Schizosaccharomyces pombe* during the meiosis I. At the onset of meiosis I, the SPBs are near each other and nucleate MTs that form the spindle. At the same time other MTs grow in other directions, having a fast turnover. We observed that some of them grow directly toward the KCs and others perform angular movement with respect to the spindle. When MTs reach a KC, the KC is captured and is subsequently retrieved to the SPB. We observed that the KCs are clustered in three pairs at the beginning of meiosis I. KCs in the same pair move in a correlated manner, but there is no correlation in the movement between KCs from different pairs. The movement of KC pairs is diffusive before a capturing event occurs, but directed afterwards. Their retrieval velocity toward the SPBs is similar to the shrinking velocity of the MTs. In order to understand the contribution of the mechanisms that lead to the observed kinetochore capture times, we developed a theoretical model. The model includes MT pivoting around the SPB (Kalinina et al., Nat Cell Biol 2013), MT dynamics and paired movement of the KCs. Our simulations show that paired movement of the KCs, in addition to dynamic MTs, can explain the observed capture times of KCs during meiosis I.

P1828**Board Number: B352****DNA segregation before mitosis: Re-entering mitosis after multiple S-phases.**B. Stormo¹, D. Fox²;¹Cell Biology, Duke University, Durham, NC, ²Pharmacology and Cancer Biology, Duke University School of Medicine, Durham, NC

The canonical cell cycle alternates rounds of DNA replication, which generate pairs of homologs held together by cohesin, with rounds of division that separate these pairs into two cells. This cycle is essential for ensuring accurate segregation of chromosomes and preventing the generation of aneuploidy. In contrast, in specific metazoan tissues including in humans, some cells undergo an alternate cell cycle termed the endocycle in which there are multiple rounds of S-phase without karyokinesis. Endocycles can also occur pathologically, including after treatment with many current cancer therapeutics, yielding aberrant mitotic divisions. These mitotic cells are known to have diplochromosomes, in which four homologous chromatids are conjoined. How diplochromosomes can be resolved without causing aneuploidy is unknown. To study this question we have used a naturally occurring population of mitotic endocycled cells. The rectal papillar cells of *Drosophila melanogaster* undergo repeated rounds of S-phase to generate DNA content of 8N then these cells undergo two full rounds of mitosis. Using a combination of time-lapse fluorescence microscopy and classical cytology we report a mechanism we term “pre-mitotic DNA segregation (PMDS).” Rather than forming persistent diplochromosomes, papillar cells instead form transient conjoined chromosomes that are accurately and elegantly separated into sister chromatid pairs prior to metaphase. We argue that PMDS prior to mitotic division is an extra step that any cell must undergo in the first division following multiple S-phases, but not in subsequent divisions. In addition to PMDS, our research also likely suggests that papillar cells periodically release chromatid cohesion in a non-canonical manner during the endocycle to prevent the formation of diplochromosomes. How these processes are controlled during the endocycle and during PMDS in papillar cells has implications for the aneuploidy described in the numerous documented cases of multiple S-phases without karyokinesis in the cancer literature.

P1829**Board Number: B353****Coordinated chromosome movement in the absence of physical connection in spider spermatocytes.**A.O. Nedo¹, C.M. Andreychik¹, R.N. Doan¹, L. Jamrog¹, L.V. Paliulis¹;¹Biology Department, Bucknell University, Lewisburg, PA

In cell division, physical connection guarantees that movement of partner chromosomes is coordinated; i.e. connected sister chromatids move together toward their associated spindle pole in anaphase I of meiosis. Interestingly, there are some cell types in which unconnected chromosomes always move together, allowing study of how chromosomes communicate their position in the cell in the absence of

physical connection. We use spiders with the $X_1X_1X_2X_2$ (female)/ X_1X_20 (male) sex determination system to study coordinated chromosome movements. In males the nonhomologous X_1 and X_2 chromosomes always associate with the same spindle pole from prometaphase I through anaphase I. After seeing a visible gap between X_1 and X_2 , we hypothesized that the gap was reflective of a lack of connection between X_1 and X_2 . We found that X_1 and X_2 are easily separable using a micromanipulation needle in metaphase I, showing that they move together in the absence of physical connection. When we used a micromanipulation needle to separate X_1 and X_2 such that they associated with opposite poles, we found that cells could progress into anaphase. This showed that cosegregation of X_1 and X_2 is not monitored by the cell following initial chromosome attachment in prometaphase I, which would seemingly lead to error-prone segregation of sex chromosomes in male meiosis. We found that consistently correct segregation of X_1 and X_2 occurs because X_1 and X_2 appear to associate with a spindle pole while in the nuclear envelope and, under our observation, never reorient during meiosis I, suggesting that continuous monitoring of the position of X_1 and X_2 is not necessary in normal meiosis I. Spindle breakdown and reassembly during prometaphase I could, however, lead to sex chromosome aneuploidy.

P1830

Board Number: B354

Mitotic chromosome alignment is dispensable for accurate segregation of the genome.

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Chromosome alignment at the equator of the mitotic spindle is a highly conserved step during cell division. This process requires spatial control of kinetochore microtubule (k-fibers) dynamics and is widely believed to promote the equal distribution of replicated sister chromatids during anaphase. However, this latter assumption has never been experimentally tested due to the difficulty of disrupting chromosome alignment without also compromising the attachments between kinetochores and spindle microtubules. We find that depletion of the kinesin-like motor Kif18A from diploid human cells significantly disrupts chromosome alignment while only modestly affecting mitotic progression. Surprisingly, loss of Kif18A does not lead to an increase in aneuploidy, suggesting that chromosome alignment is largely dispensable for accurate chromosome segregation. Instead, we find that disrupting chromosome alignment leads to defects in the organization of the nucleus during the subsequent interphase. These data suggest that mitotic chromosome alignment may function to preserve the three-dimensional organization of interphase chromosomes from cell cycle to cell cycle.

P1831**Board Number: B355****A computer program to identify meiotic recombination hotspots with unprecedented precision.**O. Miura¹, Y. Kojima¹, T. Ohyama¹;¹Biology, Waseda University, Tokyo, Japan

The meiotic recombination events occur in specific chromosomal regions. These regions are called recombination hotspots. In the yeasts *S. cerevisiae* and *S. pombe*, no motif sequence has been identified in the recombination hotspots, while in mice and humans, some motifs have been reported. However, regions besides the hotspots also contain them with no small frequency, which indicates that they are not sufficient as the marks for the formation of recombination hotspots. We have investigated the relationship between the recombination hotspots and the physical properties of DNA.

Meiotic recombination events are initiated by formation of DNA double-strand breaks (DSBs). Thus, using the genome databases of *S. cerevisiae*, *S. pombe* and *Mus musculus*, we investigated characteristics of the physical properties (e.g., flexibility, duplex disruption energy, protein-induced deformability) of DSB hotspots. When averaged, a common distinctive property was clearly detected at DSB hotspots for each organism, which allowed us to develop a computer program that can identify DSB hotspots of a given genomic DNA. When applied to *S. cerevisiae*, this program showed a high performance with the accuracy rate of identification of ~77%, suggesting that our program is a promising tool to understand the mechanism underlying the generation of the recombination hotspots.

P1832**Board Number: B356****Karyotypes of *Anopheles gambiae* s.l. in forest and arid zones of Nigeria and Cameroon.**A.M. NOUTCHA¹, C.I. Anumudu²;¹Dept of Animal Environmental Biology, University of Port Harcourt, Port Harcourt, Nigeria,²Department of Zoology, University of Ibadan, Ibadan, Nigeria

Fast and reliable recognition of malaria vectors in an area is a basic prerequisite for the utilization of appropriate malaria control strategies. Three of the 7 sibling species of the African malaria mosquito *Anopheles gambiae* s.l. are found in Nigeria and Cameroon: (*Anopheles arabiensis*, *Anophelesgambiae* s.s. and *Anopheles melas*). Of these species *Anopheles arabiensis* and *Anopheles gambiae* s.s. present polymorphic paracentric inversions in their chromosomes, which generate diverse chromosomal karyotypes. These chromosomal arrangements are thought to impart selective advantages for each form to survive in arid and forest environments. In West Africa, studies on cryptic karyotypes of *An. gambiae* s.l. have been concentrated in Mali and coastal Cameroon. This study was undertaken in two villages (Bama and Mora) of an arid zone, and two villages (Fokoué and Igbo-Ora) of a forest zone. Bama and

Igbo-Ora are in Nigeria while Mora and Fokoué are in Cameroon. Mosquito collections were made for 7 consecutive days in each month from bedrooms/houses over an 18-month period. Indoor collections were from May to October of the following year, covering two rainy and one dry seasons. Chromosomal preparations were based on a modified Coluzzi method. Species identification and chromosome scoring were undertaken with a phase-contrast microscope. Slides were analyzed under a fluorescent microscope to observe inversions. These inversions were summarized to obtain karyotypes and chromosomal forms. Among the karyotyped anophelines (n=20), 35% were *An. gambiae s.s.* and 65% were *An. arabiensis*. The karyotypes were: 15% standard +/+, 5% ab/b, 25% bc/b, 10% bc/bc, 35% b/b, 5% b/+, 5% bd/bf and varied among *An. gambiae s.l.* The standard +/+ (43%), the b/+ (14%) and bc/bc (29%) karyotypes, characterized *An. gambiae s.s.*, while b/b (53%) ab/a (8%) and bd/bf (8%) karyotypes characterized *An. arabiensis*. Variation of karyotypes across ecozones showed that 22% were caught in arid zone and 78% in the forest zone. The temporal distribution revealed that 48% were caught in the first rainy season and 52% in the dry Harmattan and second rainy seasons. The implications of the speciation process in *An. gambiae s.l.* in the epidemiology of malaria in Nigeria and Cameroon will guide their malaria control strategies.

Key Words: Paracentric Inversions, Karyotypes, Chromosomal forms, *Anopheles gambiae s.l.*, Ecozones, Nigeria-Cameroon.

P1833

Board Number: B357

Divergent kleisin subunits of cohesin specify mechanisms to tether and release meiotic chromosomes.

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Proper establishment and two-step release of meiotic sister chromatid cohesion (SCC) is critical for the production of haploid gametes. We have shown that multiple, functionally specialized cohesin complexes mediate meiotic SCC in *C. elegans*. These complexes differ in a single subunit, called the α -kleisin. Unexpectedly, the meiotic kleisins REC-8 and COH-3/4 endow cohesins with distinctive properties, specifying how complexes load onto chromosomes, become triggered to tether sisters, and then are removed from complementary chromosomal regions in late prophase of meiosis I. While stable association of REC-8 cohesin with meiotic chromosomes requires HTP-3, a component of meiotic chromosomal axes, and TIM-1, a *C. elegans* TIMELESS ortholog, COH-3/4 cohesin loads onto chromosomes independently of these factors. Once loaded, REC-8 cohesin appears to establish SCC during premeiotic S phase. In contrast, COH-3/4 cohesin becomes cohesive through a replication-independent mechanism initiated by the double-strand DNA breaks that induce crossover recombination. Break-induced SCC is thus an essential means for tethering replicated meiotic chromosomes rather than simply a specialized mechanism to repair stochastic DNA damage in proliferating cells. Later, recombination stimulates separase-independent removal of REC-8 and COH-

3/4 cohesins from reciprocal chromosomal territories that flank the crossover site. This region-specific removal occurs during late prophase and prometaphase I and likely promotes the two-step separation of homologs and sisters. Unexpectedly, we found that COH-3/4 cohesin performs cohesion-independent functions in synaptonemal complex assembly. Our findings establish new models for cohesin function in reducing genome copy number. These models diverge from those established in yeast but likely apply directly to plants and mammals, recently shown to utilize similar sets of meiotic kleisins.

P1834

Board Number: B358

Holding homologs together: centromere-centromere interactions during meiotic prophase.

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Proper chromosome segregation in meiosis is important for maintaining the fidelity of the genome from generation to generation. In humans, improper chromosome segregation during meiosis is the leading cause of infertility, miscarriage, and genetic birth defects. During prophase of meiosis in budding yeast, homologous chromosomes become tethered in a number of ways, but all of these are mediated in part by Zip1. In early prophase, centromeres undergo a period of pairing with non-homologous partner centromeres – a process called centromere coupling; the function of this process is yet unknown. As prophase progresses, homologous chromosomes find their partner and zipper together along their length by a structure called the synaptonemal complex. Zip1 is a major structural component of the synaptonemal complex; during this time Zip1 also promotes the maturation of cross-overs. In late prophase, the synaptonemal complex along the chromosome arms disassembles, but Zip1 remains at the centromeres where it tethers the homologous chromosomes together until metaphase onset. This association of the centromeres is termed centromere pairing. Recent evidence from mice suggests SYCP1, the functional homolog of Zip1, may be playing a similar role in pairing centromeres. We aim to better understand the contributions made to meiotic segregation fidelity by Zip1, especially in its roles in pairing centromeres. We have created a series of in-frame deletion mutants of ZIP1 in *Saccharomyces cerevisiae* that we are using to identify functional domains of Zip1 that are critical for centromere pairing and coupling.

P1835**Board Number: B359****Defining the mechanism by which polyploidy contributes to genomic instability.**S. Chen¹, J. Stout², B.R. Calvi¹, C.E. Walczak²;¹Department of Biology, Indiana University, Bloomington, IN, ²Medical Sciences, Indiana University, Bloomington, IN

Changes in cell cycle progression are known to lead to genomic instability. One type of variant cell cycle is the endocycle in which cells go through alternating periods of synthesis (S) and gap (G) phases without an intervening mitosis, which leads to polyploidy. Polyploid cells are one hallmark of cancer, but the mechanisms that induce polyploidy and how they contribute to oncogenesis are not well understood. Traditional anti-mitotic drug treatments were reported to induce polyploidy in cancer cells in multiple types of cancers. Here, we showed that multiple anti-mitotic drugs induced polyploidy in MDA-MB-231 breast cancer cells and in HeLa cervical carcinoma cells. Of these drugs, RO3306 (a Cdk1 inhibitor) and SU6656 (a Src kinase inhibitor) reproducibly induced a large fraction of the cells to have higher DNA content, indicative of an endocycle. We showed that cells undergo mitotic-like rounding after drug addition, but then re-flatten without completing division, resulting in large, multi-lobed nuclei. Combined with analysis of fixed cells in the rounded state showing early but not late mitotic features, we found that RO3306 and SU6656 cause a specific type of endocycle known as endomitosis. Furthermore, we analyzed cell fate after RO3306/SU6656 withdrawal by FACS, time-lapse microscopy and fixed cell analysis. We found that polyploid MDA-MB-231 cells were able to resume mitotic divisions and returned to a pseudo-diploid state after drug withdrawal. Mitotic polyploid cells with supernumerary centrosomes formed large multi-polar spindles that contained hundreds of extra chromosomes. Upon cell division there were lagging chromosomes and chromosome bridges, suggesting that mitotic errors in these cells contribute to further aneuploidy. Our results demonstrate that inhibition of normal cell cycle progression can lead to polyploidization and genome instability. Cancer cell polyploidization may represent a survival mechanism for cancer cells in response to cancer therapies and may be a major contributor to disease relapse.

P1836**Board Number: B360****Sperm DNA positioning during female meiosis in *C. elegans*.**M. Panzica¹, F. McNally¹;¹Univ California-Davis, Davis, CA

In most animals, fertilization occurs prior to the completion of female meiosis, raising the question of how the oocyte can tolerate an extra copy of the genome (the sperm DNA), while still segregating its own genomic copies. Evidence from mouse, starfish and *C. elegans* suggests that fertilization occurs at a site far from the meiotic spindle, however, some mechanism must exist to maintain the distance between the sperm and meiotic spindle.

A recent study demonstrated the importance of this distance in preventing capture of the oocyte meiotic spindle by the sperm aster when the sperm aster forms prematurely (McNally et al. 2012. *Dev. Cell* 22:788). It remains unknown if this interaction can occur independently of microtubule asters or the consequences of such an interaction. In the *C. elegans* meiotic embryo, yolk granules and sperm DNA are both moved inward in a kinesin-dependent manner. However, the sperm is restricted to the opposite end of the embryo from the meiotic spindle. We hypothesize that an actin network with a defined mesh size restricts kinesin-driven sperm movement while allowing transport of the smaller yolk granules throughout the embryo.

To test this hypothesis, zygotes in which F-actin was disrupted by depletion of profilin (PFN-1) were analyzed by fixed immunofluorescence. The average distance between the sperm and oocyte meiotic spindle in control embryos was $76.30 \pm 0.93\%$ embryo length (n=78), whereas pfn-1 (RNAi) embryos averaged $51.15 \pm 3.76\%$ embryo length (n=51), with some as close as 5% embryo length. Time-lapse imaging of meiotic embryos from worms expressing GFP-yolk protein and mCherry:histone revealed that yolk granules move rapidly over long distances whereas the sperm moved only within a confined volume at a slower velocity. In pfn-1(RNAi) embryos, the sperm moved over long distances with the yolk granules. Preliminary analysis indicates that while the trajectory of sperm DNA movement changes upon profilin depletion, the velocity does not. These results support the hypothesis that an actin meshwork restricts sperm DNA movement while allowing long-range transport of smaller yolk granules.

P1839

Board Number: B361

Chromosomes mis-segregated into micronuclei cause chromosomal instability by further mis-segregating at subsequent mitoses.

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A micronucleus (MN; plural MNi) is a small nucleus that emerges from a chromosome or chromosome fragment that fails to segregate into a daughter nucleus during cell division. Here, we specifically focused on whole-chromosome MNi and investigated the behavior of such chromosomes (mnChrs) at the mitotic cell division following MN formation. To this end, we used PtK1 cells and experimentally induced anaphase lagging chromosomes, which are known to form MNi upon mitotic exit (Cimini et al., *J. Cell Sci.*, 2002). The day following MN formation, most MNi (80%) maintained nuclear protein localization, indicating that the MN nuclear envelope preserved its integrity. Moreover, many micronucleated cells (MNed cells) underwent further mitotic division and a vast majority (89%) exhibited chromosome segregation errors. To investigate the contribution of mnChrs to such segregation errors, we used PtK1 cells expressing Photoactivatable GFP-histone H2B, and marked MNi in prophase cells, just before nuclear envelope breakdown. We found that the majority of MNed cells progressed into anaphase, but the mnChrs displayed a number of chromosome segregation defects, including sister chromatids that failed to fully separate at anaphase and co-segregated to the same

daughter cell, formation of new MNi in the daughter cells, and delayed condensation. We also found that such under-condensation could lead to lagging chromatin that became trapped by the cleavage furrow and caused cytokinesis failure. However, this was never the case for well condensed anaphase lagging chromosomes that did not come from a MN, which, instead, were invariably pushed to one side of the ingressing cleavage furrow. In conclusion, our work shows that although anaphase lagging chromosomes may initially segregate to the correct daughter cell, the MN that they form represents a severe threat to chromosomal stability at subsequent cell cycles.

P1840

Board Number: B362

Scaling chromosome compaction to cell size.

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During development in many organisms, cells reduce in size due to consecutive cell divisions without an increase in embryo volume and must adapt organelle sizes accordingly. Despite dramatic changes in cell size, the genome size is constant in all diploid cells of an organism. Thus, it is expected that during anaphase, condensed mitotic chromosomes must be half the length of the mitotic spindle to be properly segregated to each daughter cell, a length that varies according to cell size. Using high-resolution time-lapse microscopy of *C. elegans* embryos expressing H2B-GFP and γ tubulin-GFP, we developed a 3D method to measure chromosome length in living, developing embryos of different developmental stages, ranging from the 1 cell to the 16 cells stage embryos. We confirmed that prometaphase condensed chromosomes are smaller in length as cells reduce in size. In order to identify regulators of chromosome scaling, we design a large-scale RNAi screen in *C. elegans* based on a differential embryonic lethality of a sensitized worm strain compared to control N2 (wild-type). The sensitized animals have a telomeric fusion between the 2 longest chromosomes resulting in an abnormally long chromosome without changing total amount of DNA. We hypothesized those worms would be more susceptible to any defect in chromosome scaling compared to control. As a proof of concept, we depleted a major regulator of chromosome compaction, Condensin. Depletion of the condensin II complex in *C. elegans* completely abolishes chromosome compaction however there are no changes in F1 embryo viability after Condensin I depletion. We measured a 8-fold increase in lethality when we depleted condensin I in the long chromosome strain compared to control, indicating that a subtle change in chromosome compaction could be sufficient to result in increased embryonic lethality of the sensitized strain. Subsequently, we used a sub-library of the whole genome RNAi library that includes 438 genes known or predicted to be chromatin binding and/or modifying enzymes. The screen resulted in a total of 15 hits that we are currently validating using high-resolution microscopy. In summary, we are using large-scale RNAi depletion and high resolution imaging to determine the mechanisms of mitotic chromosome size regulation. Our research capitalizes on a normal developmental context to study a cell biological problem (chromosome condensation). We predict that these results will reveal fundamental concepts in chromosome biology.

P1843**Board Number: B363****Sister chromatid cohesion and chromosome condensation are separable pathways.**K. Tong¹, R.V. Skibbens¹;¹Lehigh University, Bethlehem, PA

High-fidelity chromosome segregation is critical for cell division and organism development. During the cell cycle, replicated DNA is identified as sister chromatids, condensed into chromosomes, and segregated into daughter cells. Cohesin complexes tether together sister chromatids and also promote chromosome condensation, indicating that these two processes are linked. Cohesion and condensation both require that chromatin-associated cohesins become modified by the S-phase acetyltransferase Ctf7/Eco1. Recent findings provide important insights into the mechanism through which Eco1 regulates cohesins. For instance, deletion of *RAD61/WAPL* from *eco1* mutant cells rescues the condensation defects, but not the cohesion defects, that otherwise occur in *eco1* mutant cells. These findings not only document that the role for Eco1 and cohesins in cohesion is separable from that of condensation, but contradict the notion that Rad61 functions as an "anti-establishment" cohesion factor. Like Eco1, Pds5 impacts cohesins and is required for both cohesion and condensation. Recent studies reveal that *ELG1* deletion suppresses both the temperature sensitivity of *pds5* mutant cells as well as *eco1* mutant cells. However, the mechanism through which Elg1 regulates cohesion and condensation remains unknown. Prior analyses suggest that yeast cell lethality in cohesin mutants is tied to loss of condensation – not cohesion. Here, we show that *ELG1* deletion in *pds5-1* mutant cells results in a significant rescue of cohesion, but not condensation. Thus, a rescue of cohesion in *pds5* mutants is sufficient to maintain cell viability despite persistent and profound condensation defects. Primarily, Elg1 unloads the DNA replication clamp PCNA from DNA during S phase. We show that PCNA overexpression also rescues the temperature sensitivity, but not the condensation defect, in *pds5-1* mutant cells. These findings suggest that *ELG1* deletion rescues *pds5-1* mutants through PCNA regulation. In contrast to results that *RAD61* deletion rescues both *eco1* mutant cell condensation defects and cell viability, we find that *RAD61* deletion fails to rescue *pds5-1* mutant cell temperature sensitivity – even though the condensation defect is rescued. In conclusion, we provide new evidence that Eco1 and Pds5 perform separable and distinct roles in cohesion and condensation and that both pathways impact cell viability in non-redundant mechanisms.

P1844**Board Number: B364****The unique structural features of CENP-A nucleosomes in solution are highly pronounced on α -satellite DNA sequence.**N. Sekulic¹, K. Gupta¹, G. Van Duyne¹, B.E. Black¹;¹Department of Biochemistry and Biophysics, University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA

The centromere is a locus present on each chromosome to ensure their proper segregation during cell division. Centromeres are defined by the presence of nucleosomes containing a histone H3 variant, CENP-A. Many eukaryotic centromeres are found on slightly A/T rich and repetitive DNA. In humans, centromeres typically are found on highly repetitive DNA, termed α -satellites, where the smallest repetitive unit is 171 bp. Centromeres known to exist at genomic locations without any detectable α -satellite DNA, at so-called neocentromeres. Thus, there is not an absolute requirement for any particular DNA sequence for centromere function and inheritance, but it is likely that the preponderance of these sequences at typical human centromeres is a result of a functional benefit they provide. In this context, we now explore the role of DNA sequence in the physical properties and shape of the CENP-A nucleosome in solution. Our present studies include analytical ultracentrifugation and contrast variation by small-angle X-ray and neutron scattering (SAXS/SANS). Contrast variation is a powerful approach that leverages the scattering powers of protein and DNA components in different solution environments to resolve properties of the parts from the whole. The DNA templates that we used to reconstitute nucleosome core particles were the widely used synthetic nucleosome positioning sequence, termed Widom 601, and an α -satellite sequence where the position within the α -satellite monomer corresponds precisely to what we recently mapped as the most heavily occupied site at centromeres (Hasson et al., 2013, Nat. Struct. Mol. Biol. 20:687-95). While the gross properties of canonical H3 and CENP-A nucleosomes on Widom 601 DNA were similar by sedimentation velocity analysis, contrast variation analysis reveals discrete differences in both the protein and DNA components that can be correlated with the structural features of available atomic models. When CENP-A nucleosome core particles are assembled with its native α -satellite DNA, however, we observed significant changes in the solution structure of the CENP-A nucleosome relative to on Widom 601 DNA. In sum, our findings support the notion that CENP-A confers conformational changes to both the histones and the DNA at the termini of nucleosomes and that these changes are magnified on its preferred native DNA sequence. Our findings should be considered in the ongoing effort to provide a structural framework to understand the physical basis for how CENP-A distinguishes centromeres from the rest of the genome.

P1847**Board Number: B365****Complex chromosomal rearrangements from the missegregation of intact chromosomes.**D. Pellman¹, A. Spektor¹, C. Zhang², J. Francis², M. Meyerson³;¹Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA, ²Broad Institute of MIT and Harvard, Cambridge, MA, ³Dana-Farber Cancer Institute, Boston, MA

Whole chromosome segregation errors are among the most common alterations in cancer genomes. However, whether they are cause, consequence, or neutral bystanders in malignant transformation has been debated. The debate about the significance of whole chromosome aneuploidy has been fueled by the paucity of mechanisms linking chromosome segregation errors to tumorigenesis. We previously reported that missegregation of chromosomes can result in DNA damage, if the missegregated chromosome is partitioned into abnormal nuclear structures called micronuclei. Micronuclei are well-described features of cancer cells. We proposed that localized DNA damage in micronuclei might be a mechanism to explain “chromothripsis”, a pattern of complex chromosomal rearrangements restricted to one or a few chromosomes discovered from cancer genome sequencing. To test this hypothesis, we developed an approach to combine live-cell imaging with single-cell genome sequencing (Look-seq). We used this approach to sequence pairs of daughter cells after the division of micronucleated cells. The analysis revealed complex chromosomal rearrangements restricted to the missegregated chromosome. In some cells, a single chromosome was fragmented, with different fragments being partitioned into different daughter cells. Together these findings define a new mutagenesis mechanism where errors in mitosis produce chromosome rearrangements relevant to cancer and developmental diseases.

P1849**Board Number: B366****Size matters: analyzing the rate of mis-segregation and incidence of aneuploidy in Indian and Chinese Muntjac cells.**J.M. Nicholson¹, N. Baudoin¹, D. Cimini¹;¹Biological Sciences, Virginia Bioinformatics Institute, Virginia Tech, Blacksburg, VA

Aneuploidy, the presence of an abnormal number of chromosomes, causes gross transcriptomic and proteomic imbalances due to the gain/loss of hundreds to thousands of genes. Such imbalances are typically detrimental to cell survival and proliferation, however can be beneficial to cells in certain contexts (e.g., cancer and evolution). Accordingly, mechanisms that allow cells to attenuate or tolerate the effects of aneuploidy are important for cell survival and adaptive potential. Recently, it was shown that tetraploidy can buffer the effects of aneuploidy on cells (i.e., tetraploid cells continue to divide after mis-segregation events whereas diploid cells arrest/die) (Dewhurst et al., Cancer Discov., 2014). Moreover, it is widely acknowledged that only aneuploidies for smaller, gene-poor chromosomes are

tolerated in utero in humans and it has been reported that cancer cells tend to lose smaller chromosomes (Duijf et al., *Int J Cancer*, 2012). Such observations suggest that the degree of aneuploidy (as fraction of genes lost/gained) is important to consider when evaluating the effects of aneuploidy. To investigate how cells with different karyotypic backgrounds tolerate such effects, we analyzed mis-segregation rates and karyotypic heterogeneity in Indian Muntjac ($2n=6$, very large chromosomes) and Chinese Muntjac cells ($2n=46$, small chromosomes) cells (IMJ and CMJ, respectively). These cells display dramatically different karyotypes yet are evolutionarily close relatives of one another. Indeed, there is convincing evidence that the large IMJ chromosomes evolved via numerous tandem fusion events from a common ancestor with a CMJ-like karyotype. We found that despite similar mis-segregation rates (measured as frequencies of anaphase lagging chromosomes), karyotypic heterogeneity (evaluated by chromosome counts in metaphase spreads) was primarily observed in CMJ and not IMJ cells, indicating that mis-segregation of small chromosomes (loss/gain of fewer genes) is better tolerated. We also found that a subpopulation of IMJ cells with near-tetraploid karyotype displayed significantly higher chromosome number variability compared to the diploid population, indicating that loss/gain of a chromosome out of two has a smaller impact than gain/loss of a chromosome out of four. Our data suggest that the degree of aneuploidy in cells is important and support recent findings showing that tetraploidy is one mechanism that allows cells to tolerate the gain or loss of genetic information.

P1850

Board Number: B367

Ki-67 is a PP1-interacting protein that organises the mitotic chromosome periphery.

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When the nucleolus disassembles during open mitosis, many nucleolar proteins and RNAs associate with chromosomes, establishing a perichromosomal compartment coating the chromosome periphery. At present almost nothing is known about the function of this poorly characterised compartment. During interphase, Ki-67 is predominantly localised in the cortex and dense fibrillar components of the nucleolus, but relocates to the periphery of condensed mitotic chromosomes. In this study, we report that Ki-67 is ancestral to the PP1 targeting subunit Repo-Man and is required for the assembly of the perichromosomal compartment in human cells. We go on to show that Ki-67 is a cell-cycle regulated PP1 binding protein that is involved in phospho-regulation of the nucleolar protein B23/nucleophosmin. Following siRNA depletion, Ki-67, NIFK, B23, nucleolin, and four novel chromosome periphery proteins

all fail to associate with the periphery of human chromosomes. Correlative light and electron microscopy (CLEM) analysis of cells depleted of Ki-67 suggest a near-complete loss of the entire perichromosomal compartment. Mitotic chromosome condensation and intrinsic structure appear normal in the absence of the perichromosomal compartment but significant differences in nucleolar reassembly and nuclear organisation are observed in post-mitotic cells. Remarkably, although the abnormalities in the dispersal and re-aggregation of nucleolar components during mitosis appear quite dramatic, Ki-67-depleted cells appear to survive with a single nucleolus and can proceed to another cell cycle. This second division becomes more problematic and we have observed an increase of apoptosis together with mitotic delay. The mitotic defects that occur at the second mitosis after loss of the perichromosomal layer, could be related to a necessary interphase function of Ki-67 or reflect decreased synthesis of a key component required for chromosome segregation, and therefore will require future investigation.

P1853

Board Number: B368

Regulation of multiple cohesin complexes in *Drosophila* meiosis.

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The cohesin complex is a ring-shaped protein complex that is important for holding sister chromatids and is also required for assembling synaptonemal complex (SC), a protein structure situated between meiotic homologous chromosomes. The cohesin complex consists of four subunits, SMC1, SMC3, a kleisin member (Rad21 in *Drosophila*) and SCC3 (stromalin/SA in *Drosophila*). In *Drosophila* meiosis, after knocking down SMC1 and SMC3, we found that the SC fails to form. However, when knocking down SA and Rad21, the SC phenotypes differ: SA knockdown has a patchy SC phenotype, whereas Rad21 has wild-type like phenotype. These results suggest that there is another subunit substituting for Rad21 possibly leaving Rad21 defunct in *Drosophila* meiosis. In addition, because various cohesin mutants show different SC defect phenotypes, we suggest there might be at least two types of cohesin complexes involved in *Drosophila* meiosis. One complex might include SA and C(2)M, a kleisin-like protein; the other might include SUN and SOLO. Differential regulation of these complexes might allow for separately regulating arm and centromere cohesion, required for separation of homologous chromosomes at meiosis I which maintaining sister chromatid cohesion until meiosis II. However, little is known about how these cohesin complexes are regulated and ultimately released at the right time for chromosome segregation. To examine the consequences of no cohesion during meiosis I, we generated SMC1 mutant oocytes. These oocytes showed precociously separated homologous chromosomes and more than 8 foci of centromere staining, confirming that SMC1 is required for sister cohesion at meiosis I. These results are similar to our observations of *ord* mutants, which had previously been shown to be required for proper segregation of sister chromatids. Our future work will be focused on examining the function and regulation of each cohesin complex. We will examine the phenotype of *c(2)M* and *c(2)M ord* double mutants to determine if the phenotype of losing two putative cohesin complex will be similar

to the SMC1 mutant phenotype. To examine how the different cohesin complexes are regulated during meiosis, we will examine chromosome behavior in oocytes lacking both positive (eco and Nipped-B) and negative (wapl and separase) cohesin regulators. These experiments will provide insights into how arm and centromere cohesin is regulated during the metaphase-anaphase transitions of the two divisions of meiosis.

P1854

Board Number: B369

The Use of Ellman's Reagent in the Isolation of Mitotic Chromosomes for Structural and Biochemical Studies.

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Histones H1 and H3 are highly phosphorylated during mitosis in vertebrate cultured cells, but they are rapidly dephosphorylated by endogenous protein phosphatases when the mitotic cells are lysed. This dephosphorylation can be prevented by including sulfhydryl reagents in the lysis solutions, and p-chloromercuriphenyl sulfonate (PCMPS) is particularly effective. However, the toxicity of PCMPS and the fact that it is no longer available in the U.S. led us to examine another sulfhydryl reagent, Ellman's Reagent (5,5'-dithiobis-(2-nitrobenzoate), or DTNB). DTNB reacts efficiently with sulfhydryl groups and can be used for their quantitation.

Using acid-urea polyacrylamide gel electrophoresis, which separates phosphorylated and unphosphorylated histone species, and SDS-polyacrylamide gel electrophoresis combined with phosphoprotein-specific stains or western blotting with phosphohistone-specific antibodies, we show that DTNB is very effective in preventing dephosphorylation of histones H1 and H3. The minimal effective DTNB concentration is approximately stoichiometric with the concentration of sulfhydryl groups in the lysate, and the phosphatase inhibition can be reversed by treatment with 2-mercaptoethanol or dithiothreitol. DTNB is compatible with several available procedures for the isolation of metaphase chromosomes.

Interestingly, chromosomes isolated with DTNB are "cleaner" in that they have fewer and less intense non-histone protein bands on gels. This suggests that DTNB may prevent the artefactual association of non-chromosomal proteins with the chromosomes during isolation.

P1857**Board Number: B370****Post-mitotic transcription reactivation: insights into cell identity maintenance.**K. Palozola¹, J.S. Becker¹, G. Donahue¹, J. Caravaca¹, K.S. Zaret¹;¹Institute for Regenerative Medicine, Univ Pennsylvania, Philadelphia, PA

The goal of our research is to understand how a cell accurately reinitiates its cell type-specific transcriptional program after mitotic division. During mitosis, the genome becomes transcriptionally silent. Yet, after each cell division, cells retain their identity by reinitiating their pre-mitotic, cell-type specific transcriptional program. Because a cell's gene expression profile largely determines its identity, cells may be more susceptible to reprogramming to alternate cell fates in mitosis than in interphase. Thus, understanding the mechanisms of genome-wide transcription re-initiation following mitosis would contribute to our understanding of cell identity and aid in the development of cell reprogramming technologies. During mitosis, RNA polymerase II (Pol II) and most transcription factors are excluded from mitotic chromatin. However, recent studies have identified a subset of cell type-specific transcription factors, such as FoxA1 in hepatocytes, that are retained on mitotic chromatin and promote the rapid re-activation of target genes at mitotic exit – a mechanism termed mitotic bookmarking. Yet, the timing and mechanism of genome-wide transcription re-activation has not been studied. Since the nuclear envelope is disassembled in mitosis, existing methods for labeling nascent RNAs, such as GRO-seq, are not applicable due to their reliance on the presence of the nuclear envelope. Accordingly, we have employed new methods to label nascent transcripts as cells exit mitosis. Specifically, a human hepatoma cell line, HUH7, was arrested in prometaphase, released from arrest, and the nascent transcripts were pulse-labeled with 5-ethynyluridine (EU). Subsequent isolation, and high throughput sequencing of cDNA from pulse-labeled RNA suggests that not all genes are re-activated with the same kinetics. Continuing analysis aims to understand the role of mitotic bookmarking factors and Pol II recruitment in this process.

Tumor Invasion and Metastasis 2**P1860****Board Number: B372****Role of E-cadherin cell surface activation in cancer progression.**Y. Petrova¹, B.M. Gumbiner¹;¹UVA, Charlottesville, VA

E-cadherin is a tumor suppressor protein and the loss of E-cadherin expression promotes tumor progression and metastasis. However, in many cases cells of epithelial tumors and metastases still express E-cadherin. We have found that E-cadherin exists in different activity states at the cell surface, and we hypothesize loss of cadherin activity regulation, rather than expression, may promote cancer

progression. To study whether adhesion activation can inhibit metastasis, we tested the effects of mAbs that activate E-cadherin at the cell surface. Orthotopic tumors were formed by injecting mouse mammary 4T1 cells expressing human E-cadherin into mammary fat pads of Balb/c mice. The experimental group received twice weekly intraperitoneal injections of adhesion-activating mAb, while the control group similarly received treatment with a neutral nonactivating E-cadherin-specific mAb. We found that activating Ab treatment significantly reduced the number of 4T1 mammary tumor cells that metastasized to lung after 4 weeks. We also asked whether naturally occurring missense mutations in the E-cadherin ectodomain known to promote hereditary diffuse gastric cancer (HDGC) in humans specifically affect the regulation of adhesive activity at the cell surface. To examine basic adhesive function several HDGC E-cadherin mutants were expressed in cadherin-free CHO cells. Using a quantitative flow adhesion assay, we observed that some mutants exhibited normal adhesion strength while others exhibited substantially reduced adhesion strength, although they still had greater adhesion than the totally inactive W2A cadherin mutant. To test whether the mutant E-cadherins can be activated at the cell surface they were expressed in Colo 205 cells after depletion of endogenous E-cadherin via shRNA. Parental Colo205 cells are normally nonadhesive despite having a full complement of E-cadherin-catenin complexes at the cell surface, but they can be triggered to adhere strongly, either by treatment on the outside with our activating mAbs or internally by treatment with nocodazole. Some mutants showed impaired activation upon activating mAb and nocodazole treatment even though they exhibited basic adhesion functionality in the CHO adhesion assay. Others were as sensitive to adhesion activation as wild type E-cadherin. One mutant was strongly activated from the outside by the activating mAbs, but could not be activated from the inside by nocodazole or by activating p120-catenin mutations. These findings show that regulation of E-cadherin activity on cell surface is a mechanism that contributes to cancer progression, in addition to the known mechanism of loss of E-cadherin expression.

P1861

Board Number: B373

Nuclear pyruvate kinase M2 regulates epithelial–mesenchymal transition by controlling E-cadherin expression.

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Pyruvate kinase M2 (PKM2) is an alternatively spliced variant of the pyruvate kinase gene that is preferentially expressed in embryonic development and in cancer cells. PKM2 modulates the final rate-limiting step of glycolysis and plays a key roll in establishing aerobic glycolysis in cancer cells, thereby stimulating cell proliferation and tumor growth. However, there has never been a study demonstrating

the association between PKM2 and cancer cell invasion or metastasis. Recently, PKM2 was shown to translocate to nucleus in response to EGFR stimulation and regulate gene expression including Cyclin D1 and c-Myc. Here we also report that stimulation of epithelial–mesenchymal transition (EMT), the phenomenon that occurs in cancer cells in the early stage of invasion, results in the nuclear translocation of PKM2 in colon cancer cells, which is pivotal in promoting EMT. PKM2 interacted with transforming growth factor (TGF)- β -induced factor homeobox 2 (TGIF2), a transcriptional cofactor repressor of TGF- β signaling, resulting in down-regulation of E-cadherin expression. We induced EMT to SW480 with TGF β 1 and EGF stimulation, in which PKM2 was up-regulated and the translocation to nucleus of PKM2 was observed. PKM2 inhibition using siRNA suppressed E-cadherin repression and inhibited EMT. We established SW480 with either PKM1 or PKM2 overexpression followed by EMT induction. As a result, PKM2 OE cell was more sensitive to EMT stimulation than PKM1 OE cell. IP-WB and mass spectrometry analyses showed that intranuclear PKM2 directly interacted with TGIF2 during EMT and TGIF2 inhibition with siRNA abrogated the augmented sensitivity to EMT in PKM2 OE cells. IP-WB and ChIP-qPCR showed that the interaction between PKM2 and TGIF2 recruited HDAC3 to the promoter region of E-cadherin, resulting in deacetylation of Histone H3 lysine 9 and increased expression of E-cadherin. In immunohistochemical analysis using clinical samples of colorectal cancers, PKM2 positivity correlated with lymph node metastasis and distant organ metastasis, and furthermore, associated with positive TGIF2 and negative E-cadherin expression. In conclusion, our data suggested that intranuclear PKM2 had a crucial role in promoting invasion and metastasis by genetically controlling E-cadherin expression.

P1864

Board Number: B374

Macrophages orchestrate early metastatic dissemination of pre-malignant ErbB2+ mammary epithelial cells.

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Metastases originate from tumor cells that disseminate from the primary site. This was proposed to occur only in invasive cancers. However, detection of tumor cells in the bone marrow of patients with pre-invasive breast ductal carcinoma in situ (DCIS) showed that dissemination could, through unknown mechanisms, take place earlier. Macrophages are key for mammary gland development and remodeling and they aid metastatic dissemination during late invasive stages of cancer. We therefore investigated whether these inflammatory cells might actively participate in early dissemination. Using the MMTV-ErbB2 mouse model, we show that F4/80+ macrophages associate with mammary ducts in the periphery of the myoepithelial layer but are never inside the ducts in wild type mouse mammary glands. We found that macrophages are recruited into epithelial layers of pre-malignant lesions of MMTV-ErbB2 mice. This

is dependent on the upregulation of cytokines in mammary epithelial cells induced by the amplification of the ErbB2 oncogene. Dissemination of ErbB2+ cytokeratin 8/18+ mammary epithelial cells (MECs) detected in circulation or the bone marrow was dependent on macrophages because their depletion reduced early dissemination significantly. Sites where macrophages had invaded the ducts showed disruption of the myoepithelium and reduced E-Cadherin expression in MECs. Depletion of macrophages from pre-malignant MMTV-ErbB2 mice normalized the mammary gland phenotype. Importantly, DCIS samples from patients frequently contained intra-epithelial macrophages and their presence was also correlated with reduced E-Cadherin expression in MECs. Our data suggest that macrophages actively aid early dissemination during pre-malignant stages of breast cancer. Further, when macrophages contact the luminal ductal cells, they induce a loss of E-cadherin junctions, possibly enhancing migration/invasion. Our data shifts the paradigm of metastasis by revealing that macrophage-assisted dissemination occurs much earlier than anticipated. This might be useful to better identify patients with early stage breast cancer that are at high risk of already carrying disseminated disease. More importantly, it provides a new understanding of the natural history of disseminated cancer, which is largely incurable for reasons that might be linked to early dissemination and divergent evolution of metastasis.

P1865

Board Number: B375

Linoleic acid promotes the localization of fascin in focal contacts in breast cancer cells MDA-MB-231.

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Breast cancer is a malignant proliferation of epithelial cells lining the ducts or lobules of mammary gland. Fascin is an actin binding protein of 55 kDa that plays an important role in actin organization. Particularly, it mediates the formation of cell protrusions, which mediate cell interactions and migration, as well as the formation of cytoplasmic microfilament loops that contribute to the cellular architecture and the intracellular movement. In several cancers including colon, pancreatic and breast, fascin is upregulated at both mRNA and protein, whereas its expression positively correlates with aggressivity of tumors. Linoleic acid (LA) is an essential fatty acid and the major polyunsaturated fatty acid in the diet of most humans. In breast cancer cells, LA induces proliferation, migration, invasion and the activation of signal transduction pathways. The aim of this study was to determine whether LA induces changes in the localization of fascin in breast cancer cells MDA-MB-231. Our findings demonstrate that LA induces the complex formation between focal adhesion kinase (FAK) and fascin in a time-dependent manner in MDA-MB-231 cells. Furthermore, LA also induces the localization of fascin in the focal contacts in a time-dependent manner. Since we previously demonstrated that LA induces cell migration, these findings strongly suggest that fascin plays an important role in cell migration induced by LA in MDA-MB-231 cells.

P1868**Board Number: B376****Targeting angiogenic switch for development of novel biomarker for early detection of pancreatic cancer.**

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Background: There are no warning signs or accurate, quantitative biomarkers to detect pancreatic cancer (PC) at early stages. 5 year survival rate for patients with PC is less than 5%, and complete remission is rare. Early detection increases the chances of saving more lives with the recently developed treatment plans. Studies have reported that an angiogenic switch, a shift of balance between pro-angiogenic and angiogenic state is a hallmark of cancer progression. The objective of this study was to investigate whether increased angiogenesis could be a predictor of PC occurrence and progression.

Methods: We performed Immunohistochemistry and immunofluorescence staining and quantitatively assessed sub cellular protein expression of angiogenesis associated markers such as vascular endothelial growth factor (VEGF), VEGF receptor 1 (FLT-1), VEGF receptor 2 (FLK-1), neuropilin (NP-1), cytoskeletal protein filamin A (fila), a stress inducible catalytic enzyme heme oxygenase 1 (HMOX-1) and its constitutive isoform heme oxygenase 2 (HMOX-2) in human patient samples during development and progression of PC and examined their correlations with selected clinico-pathological variables. Statistical analyses were performed using SAS version 9.2.

Results: VEGF and other proteins were stained predominantly in the membrane / cytoplasm compartment of malignant epithelium and were over expressed in tumors versus adjacent stroma. Normal epithelium showed lower expression compared to tumor. Disease stage was highly prognostic for outcome. A significant association was found between high expressions of fila ($p < 0.0001$) and HO2 ($p < 0.0005$) at earlier stage. Fila and HMOX2 showed higher expression at earlier stages of PC development, whereas others showed consistent positive correlation with progression of disease including for overall expression HMOX-1 (0.02) and NP-1 (0.049). Using total expression within the tumor, high expressers consistently correlated with worse outcomes and decreased overall survival. These data have been confirmed by serum analyses.

Conclusions: VEGF, VEGFR2, NP-1, HMOX-1, HMOX-2 and fila are expressed at varying degrees in PC and are higher in tumors versus stroma. Higher expression of HMOX-2 and fila showed correlation with earlier stage of PC development. The prognostic value of these candidate biomarkers within the tumor microenvironment may facilitate the development of a much needed early stage biomarker and a potential therapeutic target for PC.

P1869**Board Number: B377****Dynamics and function of the acto-myosin cytoskeleton and apical endocytosis during junctional disassembly in Epithelial to Mesenchyme Transitions.**S. Simoes¹, R. Fernandez-Gonzalez^{2,3,4}, U. Tepass⁵;

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Epithelial to mesenchyme transitions (EMT) are ubiquitous during animal development, allowing the formation and migration of new cell types and tissues. EMTs are also associated with cell escape during epithelial tumor progression, yet, the mechanisms responsible for triggering this process remain poorly understood. Hallmarks of these cell transitions include the disassembly of E-cadherin-based cell-cell junctions, the disappearance of the apical membrane, loss of apical-basal polarity, and acquisition of stem cell or migratory capacity. Using a novel in vivo model – the delaminating neuroblasts of the *Drosophila* embryo – and applying quantitative live imaging and cell segmentation techniques, we are studying the molecular mechanisms involved in the early stages of EMT. First, we demonstrate that EMT can occur without the transcriptional downregulation of E-cadherin, suggesting alternative driving forces for this process. Through live imaging with high temporal and spatial resolution, we found that the disassembly of junctions during cell delamination follows a planar polarized pattern, correlating with the sequential recruitment of the actin motor myosin II to anterior-posterior and dorso-ventral junctions as they disassemble. Delamination of neuroblasts is accompanied by the enrichment of Clathrin, a marker of endocytosis, at the junctions and free apical membrane and by the internalization of the apical determinant Crumbs, an upstream regulator of E-cadherin and regulator of apical membrane size. Finally, as junctions disassemble, the free, medial apical membrane decreases its area through sequential cycles of expansion and contraction, correlating with local pulses of myosin II accumulation, which may drive and stabilize contractions. These observations suggest that the loss of the apical domain during EMT is triggered by the concerted activation of the actomyosin cytoskeleton and the endocytic machinery at two apical membrane domains, the cell-cell junctions and the medial apical membrane. We are currently exploring how post-transcriptional modes of E-cadherin regulation, including the internalization of Crumbs, could play a role in junctional destabilization during this process.

P1871**Board Number: B378****Targeting pancreatic cancer metastasis through a specific inhibition of the Rac activator Vav1, a driver of tumor cell invasion.**G.L. Razidlo¹, C. Magnine¹, A. Sletten¹, M. Fernandez-Zapico¹, B. Ji¹, M.A. McNiven¹;¹Mayo Clinic, Rochester, MN

While many therapeutic approaches focus on tumor growth, metastasis is a primary factor contributing to cancer lethality. Therefore, novel therapies to target metastatic invasion could prevent tumor spread and recurrence resulting from local and distant metastasis. The protein Vav1 is aberrantly expressed in over half of pancreatic cancers, and we have found that its expression leads to enhanced invasion and migration. Vav1 is a guanine nucleotide exchange factor (GEF) that regulates invasion by promoting activation of Rac and Cdc42 to induce the formation of lamellipodia and invadopodia. Even with the expression of multiple Rho family GEFs, tumor cells that upregulate Vav1 appear to become dependent upon this exchange factor, implicating Vav1 as a potent target for therapeutic intervention. Azathioprine, well-known an anti-inflammatory compound, was recently shown to function by inhibiting Vav1/Rac signaling in immune cells. We therefore hypothesized that azathioprine could also inhibit Vav1 in pancreatic tumor cells to reduce its pro-invasive functions. Indeed, we have found that treatment of cultured pancreatic tumor cells with azathioprine inhibited invasive cell migration and matrix degradation, processes regulated by Vav1, through inhibition of Rac and Cdc42 signaling. Further, azathioprine treatment decreased metastasis in both xenograft and genetic mouse models of pancreatic cancer. Strikingly, metastasis was essentially blocked in Vav1-expressing tumors in a genetic mouse model of pancreatic cancer (p48 Cre, KRasG12D/+, p53loxP/+). These inhibitory effects were mediated through Vav1, as Vav1-negative cell lines and tumors were largely resistant to azathioprine treatment. These data extend the cell biological findings of Vav1-dependent invasion and migration into pre-clinical models of pancreatic cancer, and uncover a surprising role for azathioprine as a potent anti-metastatic agent for Vav1-positive pancreatic tumors. Supported by R01 CA104125 and R03 CA155778.

P1872**Board Number: B379****Formin Activity is Required for Branching Morphogenesis by MDCK Acini.**T. Fessenden^{1,2}, M.L. Gardel³, M.X. Benítez-Jones⁴;¹Cancer Biology, University of Chicago, Chicago, IL, ²Institute for Biophysical Dynamics, University of Chicago, Chicago, IL, ³James Franck Institute, Institute for Biophysical Dynamics and Physics Department, University of Chicago, Chicago, IL, ⁴Department of Physics, University of New Mexico, Albuquerque, NM

Epithelial tissues harness diverse cytoskeletal dynamics both for shape maintenance and morphogenic changes that occur during development. Misregulation of these processes lies at the foundation of many disease states including cancer metastasis, which requires polarized cytoskeletal activity to drive invasion. We investigated this phenomenon by analyzing the dynamics controlling branching

morphogenesis of Manin-Darby Canine Kidney (MDCK) cell acini embedded in collagen gels in response to hepatocyte growth factor (HGF). When exposed to HGF, all cells within the acinus underwent shape changes and moved extensively within the plane of the acinus. Very few cells, however, formed stable protrusions into the collagen matrix, which marks the initial stage of branching. The number of protrusions could be modified by changes to ECM composition and architecture, with enhanced collagen bundling and reduced basement membrane formation significantly increasing the number of protrusions. Inhibition of Arp2/3, which impairs lamellipodial activity, altered branch morphology without changing the frequency of protrusion. More surprisingly, formin inhibition abolished all protrusive activity into the surrounding ECM, but did not prevent cell shape changes or motility within the plane of the acinus. We also found that formin activity was necessary for single MDCK cells to establish protrusions in 3D collagen matrices. In contrast, 2D migration and wound healing did not require formin activity. Notably, however, formin inhibition reduced the formation of leader cells along the cell front during wound healing in 2D. This altered collective behavior also resulted in reduced curvature along the cell front. These results demonstrate a previously unappreciated role for formins in establishing highly polarized protrusions in single and multicellular contexts, and highlight differences in requirements for migration in 2D and 3D. Our findings have important implications for controlling phenotypes that require polarized protrusions, such as the invasion and migration of cells during cancer metastasis.

P1873

Board Number: B380

Macrophage-dependent activation of a non-canonical NOTCH-RhoA signaling pathway regulates tumor cell intravasation.

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The tumor microenvironment plays an important role during tumor progression and metastasis. Immune cells have been shown to play a pro-tumorigenic role during several steps of the metastatic cascade. In particular, tumor-associated macrophages facilitate tumor cell invasion and intravasation in vitro and in vivo. Recently, our group has shown that heterotypic cell contact between tumor cells and macrophages induce the formation of invadopodia in tumor cells, invasive structures necessary for matrix degradation and tumor cell intravasation. Using high resolution FRET imaging, we further found that macrophage-induced invadopodium formation is dependent on RhoA activation. However, what remained to be determined was the signaling pathway that regulated this heterotypic cell contact-mediated phenomenon. NOTCH signaling is known to be involved in homotypic cell-cell communication, and has recently been shown to be involved in invadopodium formation (Diaz et al., 2013). Thus, we explored the role of NOTCH in mediating macrophage-dependent tumor cell invadopodium formation. We found that upon NOTCH depletion the number of invadopodia in tumor cells remains unchanged. But surprisingly, in the absence of Notch signaling, macrophage-induced invadopodium formation and tumor cell intravasation are abolished. Moreover, RhoA is no longer activated in tumor cells upon

macrophage contact when NOTCH signaling is perturbed. These results suggest that NOTCH signaling regulates heterotypic cell contact mediated invadopodium formation through RhoA activation, and reveals a novel mechanism for both invadopodium formation and the NOTCH signaling pathway.

P1874

Board Number: B381

Netrin-1 induced activation of Notch signaling regulates glioblastoma cell pathogenity.

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Glioblastoma multiforme is the most severe human brain cancer. It is incurable: the expected lifespan of patients after diagnosis is less than two years. The main reasons for the lethality of glioblastoma are its heterogeneity and resistance to all current therapies and highly invasive nature. Axonal guidance protein, netrin-1, is overexpressed in glioblastoma. This secreted, laminin related extracellular matrix protein has been previously observed to act as a survival factor for aggressive neuroblastoma, metastatic breast cancer and non small cell lung cancer.

We observed that in glioblastoma tumor biopsies netrin-1 localized to hypoxic pseudopalisade structures around necrotic tumor areas. Furthermore netrin-1 increased glioblastoma cell invasiveness in *in vitro* invasion assays and in *in vivo* intracranial orthotopic tumor xenografts. Using tandem affinity purification and mass spectrometry protein identification we found that netrin-1 forms a complex with both Notch2 and Jagged1. Recombinant Netrin-1 colocalized with Jagged1 and Notch2 at the cell surface and was further found in the intracellular vesicles with Jagged1, but not with Notch2. Netrin-1 activated Notch signaling and subsequent glioblastoma cell invasion. Interestingly, the recombinant central domain of netrin-1 counteracted the effects of the full-length netrin-1: it inhibited glioblastoma cell invasion and Notch activation by retaining the Notch signaling complex at the cell surface. This finding may have therapeutic implications. Current results reveal a new mechanism leading to glioblastoma cell invasion, where netrin-1 activates Notch signaling.

P1875

Board Number: B382

Musashi-2 (Msi-2) drives metastasis of non-small cell lung cancer (NSCLC).

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Approximately 7% of individuals born in the United States in 2013 will ultimately be diagnosed with lung cancer, and ~160,000 in the US die from this disease each year, with the majority succumbing to metastatic disease. To better understand and therapeutically manage the most common form of lung cancer, non-small cell lung cancer (NSCLC), we first used orthotopic intrathoracic injection to analyze the metastatic potential of 14 murine lung adenocarcinoma cell lines derived from tumors of *Kras*^{LA1/+}; *p53*^{R172HdeltaG/+} mice. Expression of the Musashi-2 (*Msi-2*) stem cell-associated gene was consistently elevated on both the mRNA and protein level in the most highly metastatic cancer cells. *Msi-2* is an mRNA binding protein that regulates translation, and known to be upregulated and functionally important in hematologic malignancies (e.g., CML and AML). In orthotopic lung injections of these cells in immunocompetent 129Sv mice, *Msi-2* depletion dramatically decreased invasion of mediastinal lymph nodes and abrogated metastasis. Using immunohistochemical analysis of 120 specimens of human NSCLC, we demonstrated a statistically highly significant elevation of *Msi-2* in tumor versus normal lung tissue, suggesting relevance of *Msi-2* to human cancers. We found *Msi-2* knockdown in two independent mouse NSCLC cell lines abated matrigel invasion, and decreased the rate of cell attachment. Unexpectedly, *Msi-2* knockdown also resulted in a more mesenchymal morphology, suggesting a non-canonical action in control of cell migration and invasion. We therefore used reverse phase protein array (RPPA) screening and a candidate approach to dissect the mechanism of *Msi-2* action in NSCLC metastasis. *Msi-2* knockdown elevates translation of the Numb and TGF-beta receptor 1 proteins in mouse and human NSCLC adenocarcinoma cell lines, positioning *Msi-2* to provide direct input into two signaling cascades strongly associated with epithelial-mesenchymal transition (EMT) and metastasis. Further, *Msi-2* knockdown strongly induced both the protein and mRNA expression of fibronectin 1 (FN1), an important component of the extracellular matrix is frequently the target of remodeling in EMT. Taken together, our results for the first time define *Msi-2* as a promoter of NSCLC metastasis, and suggest that *Msi-2* may play a role in EMT process by coordinately modulating the TGF-beta, NUMB/NOTCH, and ECM/integrin pathways in NSCLC.

P1876**Board Number: B383****GPCR Signaling to p110 β is Required for Invasion and Metastasis of Breast Cancer Cells.**B.D. Khalil¹, W. Abi Saab¹, Y. Wang², A.R. Bresnick³, J.M. Backer¹;¹Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY, ²Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY, ³Biochemistry, Albert Einstein College of Medicine, Bronx, NY

Phosphoinositide 3-kinases (PI3Ks) mediate cell growth, survival, motility, and responses to changes in nutritional conditions, and PI3K signaling is commonly amplified in cancer. Among Class IA PI3Ks, the p110 β isoform is unique in that it is activated by both receptor tyrosine kinases (RTKs) and G-protein-coupled receptors (GPCRs). There is strong evidence implicating chemokines and their associated GPCRs in breast cancer progression and metastasis. We recently identified the G $\beta\gamma$ binding site on p110 β and defined mutants that do not affect p110 β activation by RTKs but block activation by GPCRs. To test the role of GPCR signaling to p110 β in breast cancer, we knocked down endogenous p110 β in MDA-MB-231 breast cancer cells and replaced it with wild type, kinase-dead or GPCR-uncoupled p110 β . Compared to cells expressing wild-type p110 β , MDA-MB-231 cells expressing GPCR-uncoupled p110 β showed decreased Akt phosphorylation and chemotaxis in response to lysophosphatidic acid. Similarly, these cells were defective in macrophage-stimulated in vitro invasion and matrix degradation. In murine orthotopic xenografts, cells expressing kinase-dead or GPCR-uncoupled p110 β grew at the same rate as cells expressing wild type p110 β . However, mice with GPCR-uncoupled p110 β tumors showed lower tumor cell blood burden than mice with wild-type p110 β tumors. Cells expressing GPCR-uncoupled p110 β were defective in establishing lung metastases in a tail vein experimental metastasis assay, and showed decreased extravasation in an in vitro transendothelial migration assay. These data show that GPCR signaling to p110 β is crucial for breast cancer cells to extravasate from blood to target organs. Thus, interfering with GPCR signaling to p110 β may constitute an effective strategy in targeting breast cancer progression and metastasis.

P1877**Board Number: B384****The upregulation of PTX3 is associated with the metastatic potential of gastric cancer.**S. Kim¹, B. Choi¹, E. CHANG²;¹University of ulsan college of medicine, Seoul, Korea, ²Department of Biomedical Sciences, University of Ulsan College of Medicine, Seoul, Korea

Long pentraxin 3 (PTX3), also known as TNF-inducible gene 14 protein (TSG-14), is an important mediator of tumor-associated inflammation and affects the severity of tumor malignancies but its exact

role in the progression of gastric cancer remains unclear. In our current study, PTX3 expression was found to be upregulated in metastasized gastric cancer compared with early-stage, non-metastasized gastric cancer in human patients. Most especially, PTX3 expression was prominent and surged during late stage III in comparison with early-stage (I, II) cancer, and the increase in PTX3 expression was also confirmed in a metastatic gastric cancer cell line. PTX3 expression was found to be induced by the pro-inflammatory cytokine TNF α . Exogenous PTX3 treatment promoted the migration of metastatic gastric cancer cells and the chemotaxis of macrophages to gastric cancer cells. More importantly, PTX3 treatment enhanced interactions between gastric cancer cells and osteoblasts, which are key components of the cellular niche in bone required by metastatic cancer cells. In addition, PTX3 knockdown using PTX3-specific siRNA hampered gastric cancer cell migration, the migratory capacity of macrophages, and interactions between gastric cancer cells and osteoblasts. Taken together, these findings suggest that elevated PTX3 expression is correlated with the recurrence and metastasis associated with gastric cancer by enhancing migratory potential, thereby verifying the key role PTX3 plays in the inflammation-associated metastasis of gastric cancer.

P1878

Board Number: B385

Wnt5A promotes an adaptive, senescent-like stress response, while continuing to drive invasion in melanoma cells.

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We describe a previously unexplored adaptive stress response driven by Wnt5A in highly invasive melanoma cells. We show that highly invasive melanoma cells express functional p53 and p21, and consequently undergo a G2/M growth arrest when exposed to stress such as irradiation and BRAFV600E targeted therapy. This arrest heralds the onset of an increase of senescence markers including senescence-associated beta-galactosidase activity (SA-beta-gal), senescence-associated heterochromatic foci (SAHF), promyelocytic bodies (PML) and modified chromatin, as defined by the presence of the Histone H3 trimethyl Lys9 (H3K9Me) marks. These cells, which are positive for senescence markers retain invasive capacity, and are capable of forming new colonies, both in vivo and in vitro. Silencing Wnt5A reduces expression of these senescence markers and decreases invasiveness. Mechanistically, we show that this is governed by the non-canonical Wnt molecule, Wnt5A via p21. It is these Wnt5A high, highly metastatic cells that undergo a senescent-like arrest upon induction of stress, yet retain all the hallmarks and capabilities of very aggressive cells. We propose that this adaptive stress response

may be a way for tumors to evade genotoxic damage and targeted therapy, and may select for a subpopulation of cells, which are highly invasive. Importantly, Wnt5A is emerging as a biomarker of not only metastasis in melanoma, but also of therapy resistance.

P1879

Board Number: B386

The RNA helicase Mov10 regulates Wnt5a secretion by modulating lipid synthesis in melanoma.

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The noncanonical Wnt ligand Wnt5a is elevated in melanoma and is thought to play an important role in promoting metastasis. The molecular mechanisms leading to increased Wnt5a expression in melanoma are still unknown. We examined the levels of Wnt5a mRNA and protein in whole cell lysates as well as the level of secreted Wnt5a protein in different melanoma cell lines. We found that the level of secreted Wnt5a does not correlate with the mRNA or cellular Wnt5a protein levels. Furthermore, the invasiveness of melanoma cell lines correlates more strongly with secreted Wnt5a levels rather than the Wnt5a protein level in cell lysates. These results indicate that Wnt5a expression is regulated post-transcriptionally and that Wnt5a secretion is an essential regulatory step to promote cell invasion in melanoma cells.

We investigated several candidate proteins involved in post-transcriptional gene regulation as possible regulators of Wnt5a production. Our results indicate that the RNA helicase Mov10 plays a critical role in regulating Wnt5a modification and secretion. Mov10 forms a complex with Ago2, a component of the RNA Induced Silencing Complex (RISC), and is required for miRNA-mediated translational repression and mRNA cleavage. Mov10 also interacts with the RNA helicase UPF1 that functions in nonsense mediated mRNA decay. IHC staining of malignant melanoma tumors reveal reduced expression of Mov10 compared to benign nevi. Inhibition of Mov10 by shRNA in melanoma cells caused an increase in cell invasion *in vitro* which was dependent on the Wnt5a receptor Ror2. Inhibition of Mov10 also increased Wnt5a secretion. The efficient secretion of Wnt5a requires a posttranslational modification with the lipid palmitoleic acid and inhibition of Mov10 increases Wnt5a lipidation. Fatty acid synthase (FASN) and stearoyl-CoA desaturase (SCD) function to synthesize palmitoleoyl-CoA. Inhibition of Mov10 increases the levels of FASN and SCD resulting in increased levels of palmitoleoyl-CoA. Consistent with the idea that elevation of FASN and SCD expression is a possible mechanism for increased Wnt5a secretion pharmacological inhibition of SCD or FASN blocked the shMov10 induced Wnt5a secretion. Mov10 regulation of FASN and SCD expression appears to be direct as Mov10 interacts with the mRNAs encoding FASN and SCD in a Wnt5a dependent manner. Mov10 also interacts with Dvl2, a key component of Wnt signaling, and this interaction is also increased by Wnt5a treatment. These results uncover a novel role for Mov10 in inhibiting Wnt5a secretion through the regulation of lipid biosynthesis in melanoma.

P1880**Board Number: B387****PEAK1 kinase acts as a molecular switch to mediate TGFbeta-induced EMT in breast cancer.**M. Agajanian¹, A. Campeau¹, D. Brambilla¹, M. Hoover¹, R. Klemke², J. Kelber¹;¹Biology, California State University, Northridge, Northridge, CA, ²Pathology, University of California, San Diego, La Jolla, CA

Epithelial to mesenchymal transition (EMT) is essential and strictly regulated during normal development and tissue homeostasis. However, EMT is deregulated during progression of epithelial cancers to promote metastasis. TGFbeta is a well-characterized inducer of EMT within the tumor microenvironment, but the molecular mechanisms by which TGFbeta switches from a tumor suppressor to EMT inducer remain to be fully elucidated. We previously identified PEAK1 as a new non-receptor tyrosine kinase that associates with the cytoskeleton; binds and facilitates signaling of HER2/Src complexes; and promotes tumor growth, metastasis and therapy resistance in human cancers downstream of oncogenic Ras. PEAK1 has also been reported to promote Src-dependent focal adhesion dynamics, Grb2/Shc signaling downstream of EGFR and EMT in mammary epithelial cells. In the current study, we analyzed PEAK1 expression across published human cancer microarrays and found that PEAK1 expression in breast tumor and stromal tissues correlated with poor disease prognosis. Additionally, we observed that PEAK1 expression increased in HRas-transformed MCF10A or Neu-transformed HMLE cells during EMT induction via in vivo propagation (MCF10CA1h cells) or in vitro exposure to exogenous TGFbeta (HMLE-Neu+T), respectively. To evaluate the role of PEAK1 in TGFbeta-induced EMT, we generated empty vector (V) and PEAK1-overexpressing (P) MCF7 breast cancer cells. Overexpression of PEAK1 induced proliferation, Smad2/3 and Erk1/2 activation, and increased Src expression. It has been previously reported that TGFbeta-induced EMT in breast cancer cells invokes non-canonical Src/Grb2/MAPK pathways and depends upon extracellular substrates. Therefore, we chronically treated MCF7-V and -P cells with TGFbeta and exposed them to various ECM proteins. While TGFbeta was unable to induce EMT in MCF7 cells on plastic or in the absence of overexpressed PEAK1, TGFbeta treatment and PEAK1 overexpression cooperatively induced EMT and cell migration velocity/displacement on the ECM component fibronectin. Importantly, under these same conditions PEAK1 overexpression enabled TGFbeta to activate non-canonical Src and Erk1/2 signaling. These studies are the first to provide evidence that PEAK1 kinase mediates signaling cross talk between TGFbeta receptors and integrin/Src/Grb2/MAPK pathways. With this important information about PEAK1 function and mechanism, unique methods and reagents may be developed to target PEAK1 expression/function or upstream/downstream pathways to abrogate breast cancer progression.

P1881**Board Number: B388****Effect of cystatin C construct clones on B16 melanoma in vitro cell behavior.**S. McIntire¹, J.L. Cox¹;¹Biochemistry, AT Still University, Kirksville, MO

Metastasis is the cause of most cancer related morbidity. A naturally occurring cysteine protease inhibitor, cystatin C, has been reported to inhibit tumor cell metastasis and migration for several different cancers, however, the mechanism is still unknown. Overexpression of cystatin C results in reduced lung seeding of secondary tumors for the metastatic B16F10 cell line. Objective: Our study focused on determining which region of cystatin C is responsible for anti-metastatic action by characterizing specific constructs of cystatin C in melanoma cells. We hypothesized cystatin C inhibits melanoma metastasis through a different mechanism than directly interfering with cysteine protease activity. In one construct, the N-terminal peptide amino acids 1-10, required for cysteine protease inhibition, were deleted. In the second construct the conserved motif QLVAG was altered to GGGGG. Methods and Results: Net proliferation, migration and invasion of the cystatin C constructs were assessed. The modified Boyden chamber revealed 75% reduced invasion of N-truncated clones compared to control B16F10. A scratch migration assay showed a three-fold reduction in migration rate. The QLVAG sequence was found to be required for inhibition of B16F10 invasion and migration. Net proliferation, as measured by MTT assay, remained constant between clones. Conclusions: Overexpression of cystatin C inhibits the migration and invasion, but not proliferation, of B16F10 melanoma cells. The conserved cystatin sequence, QVVAG, is required for cystatin actions on B16 melanoma cells.

P1882**Board Number: B389****Glycosylation of cyclooxygenase-2 (COX-2) influences the migratory and invasive potential of cells.**F.S. Algaly¹, J. Hand, M.B. Sevigny¹;¹Natural Science and Mathematics, Dominican University of California, San Rafael, CA

Prostaglandins are bioactive lipids involved in many physiological functions such as maintenance of the cardiovascular, immune, renal, and central nervous systems. They also play a role in certain diseases like arthritis, cancer, and Alzheimer's. Cyclooxygenase-2 (COX-2) is the enzyme that catalyzes the initial rate-limiting step in the pathway that converts arachidonic acid to prostaglandins. COX-2 exists as two glycoforms with the molecular weights of 72 and 74 kDa, the latter resulting from the addition of a high mannose chain to the Asn580 residue ~50% of the time. The over-expression of COX-2 is believed to be linked to cancer progression and specifically appears to promote the metastatic phenotype. The objective of this study is to determine the effect of the variable glycosylation of COX-2 at Asn580 on the migratory and invasive potential of cells. COS-1 cells and the breast cancer cell line MCF7 were first

transfected with either the wild-type or Asn580-mutant human COX-2 gene. Boyden chambers were used to determine the ability of transfected cells to migrate through the membrane, and modified Boyden chambers (BioCoat Matrigel Invasion Chambers) were used to test cells' invasive potential. Approximately 5×10^4 cells were plated onto the chambers, and cells were incubated for 16-18 h. Cells were then fixed, stained, visualized under a light microscope, and counted. In a previous study, our lab showed that COS-1 cells transfected with the Asn580-mutant COX-2 gene migrated faster through the membrane. In this current study, COS-1 cells transfected with the Asn580-mutant COX-2 gene also had a greater invasive potential; however, MCF7 cells transfected with the wild-type human COX-2 gene migrated faster. The results indicate that the ability of this additional glycosylation of COX-2 at Asn580 to either enhance or inhibit the migratory and invasive potential of cells depends greatly on cell type. To confirm this, future studies will be carried out to determine the effect of COX-2 glycosylation on the invasive potential of MCF7 cells as well as the migratory and invasive potential of other cancer cell lines such as PC-3.

P1883

Board Number: B390

HPSE 1 expression correlates with the invasive potential of DU145 prostate tumor cells.

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The prostate gland is target of several disorders including prostatitis, benign hyperplasia and cancer. Together, these diseases are responsible for a large fraction of discomfort and non-accidental deaths among men with advancing age. The knowledge of regulatory mechanisms underlying prostatic diseases would be important to better understand prostate growth and physiology. The degradation of basement membrane components and other molecules of the extracellular matrix is a critical step of the cascade events leading to metastasis. Tumor cells degrade these components using a variety of enzymes such as heparanase-1 (HPSE1) and matrix metalloproteinases (MMPs). The HPSE1 is an endoglycosidase that degrades heparan sulfate chains. HPSE1 overexpression is closely related with tumor reduced postoperative patients affected by cancer processes, metastatic potential, tumor vascularity and survival. This prompted us to modulate HPSE1 expression in a prostate cell line (DU145), to determine the effect of this modulation on invasive phenotype. DU145 cells had HPSE1 either overexpressed or depleted by shRNA. Parental cells served as controls. Cells in these different conditions were evaluated for expression of HPSE1 and other molecules related to adhesion, invasion and invadopodia activity. Fluorescent substrate degradation assay showed that the HPSE1 overexpressing clone presented HPSE1 expression colocalized with areas of substrate degradation, suggesting that heparanase would be involved with invadopodia activity. Furthermore, immunoblot showed that overexpression of HPSE1 increased levels of cortactin, a protein related to invadopodia formation and the metalloproteinase

MT1-MMP. Overexpression of HSPE1 increased invasive activity of DU145 cells, as shown in Boyden chambers coated with Matrigel. We addressed signaling mechanisms related to HSPE1 effects. Immunoblot showed that both total and phosphorylated FAK correlated with HPSE1 expression levels. We conclude that HPSE1 overexpression correlates with invasive potential of DU145 prostate tumor cells. Moreover, HPSE1 assembles with other membrane receptors/enzymes at the sites of matrix degradation. Financial support from CNPq (Universal 478813/2011-2) and FAPESP (Grants 2011/08559-1 and 2009/16150-6).

P1884

Board Number: B391

Expression and subcellular distribution of β -dystroglycan and nuclear envelope proteins in prostate cancer.

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The dystrophin glycoprotein complex, DAPC is a membranal protein complex involved in the structure and function of the plasma membrane during the muscle contraction-relaxation process. Key proteins of this complex are α - and β -dystroglycan, which provide a link between the extracellular matrix and the cyto skeleton through their interaction with extracellular matrix and cytoskeleton proteins respectively. β -dystroglycan is imported to the nucleus via recognition of its nuclear localization signal (NLS) by importins. Nuclear β -dystroglycan interacts with the nuclear envelope proteins emerin and lamins A/C and B1. β -dystroglycan has a molecular weight of 43 kDa, but a 30 kDa fragment is generated through its proteolytic cleavage by the extracellular matrix metalloprotease MMP9.

β -dystroglycan and lamins A/C and B1 have recently been related to certain types of cancers. Particularly, nuclear accumulating of 30 kDa β -dystroglycan as well as an altered expression of lamins B1 and A/C have been revealed in prostate cancer. In the present study we analyzed the subcellular distribution of β -dystroglycan in different prostate cancer cell lines with varying degrees of invasiveness, using immunofluorescence assays. We noted that β -dystroglycan accumulates in the nucleus and nucleolus of the cancer cells with less invasiveness, and interestingly, the nuclear localization of β -dystroglycan disappeared in the more invasive cells. Then, we will examine whether the gain and subsequent loss of the nuclear function of β -dystroglycan is related to an aberrant function of nuclear envelope proteins such as emerin and lamins A/C and B1 during prostate cancer progression.

P1885**Board Number: B392****Role of Keratin 8 phosphorylation in neoplastic progression of squamous cell carcinoma.**R. Tiwari¹, H. Alam², M.M. Vaidya³;

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Keratins were thought to be mere structural proteins whose known function was maintenance of structural integrity of cell. But recent progress in the field of keratin biology has indicated that, they do have key regulatory functions. We have previously shown role of Keratin 8 and 18 in malignant transformation in stratified epithelial cells (Raul, Sawant et al. 2004). Results from previous study in our lab suggests that K8 and K18 promote cell motility and tumor progression, possibly by deregulating $\beta 4$ integrin signaling in OSCC (Alam, Kundu et al. 2011). K8/18 undergoes several post translational modifications including phosphorylation, which is known to regulate various cellular processes; however its significance in neoplastic progression is still emerging. Our mutational studies showed that loss of K8 phosphorylation leads to increased migration and tumorigenicity (Alam, Gangadaran et al. 2011) in OSCC cells although the mechanism responsible for these phenotypes is still largely unknown. Next we wanted to investigate role of K8 phosphorylation in skin epidermoid carcinoma cell line (A431) at phenotypic and molecular levels. In order to address this question we have stably knocked down Keratin 8 in A431 cells which led to decrease in tumorigenic potential of the cell like migration and soft agar colony formation. Further in K8 null background shRNA resistant flag tagged K8 wild type, Phospho-dead and phospho-mimetic mutants were stably over expressed. To our surprise A431 cells showed significant decrease (2 fold) in cell migratory and invasive phenotype of phospho-dead clones in comparison to wild type. Contrary to that phospho-mimetic clones showed significantly more migratory and invasive behaviour compared to phospho-dead clones (1.5 folds) and is more towards wild type. To further validate our results in vivo we are investigating the role of K8 phosphorylation using transgenic mouse model system. In this direction we are generating tissue specific transgenic mouse expressing K8 wild type and phospho-mutants. We are also investigating the molecular basis for these changes related to K8 phospho- mutants in skin carcinoma derived cells. These studies would help us to better understand the role of K8 phosphorylation in tumour progression of SCC.

P1886**Board Number: B393****ELK3 expression correlates with cell migration, invasion and membrane type 1-matrix metalloproteinase expression in MDA-MB-231 breast cancer cells.**K. Park¹, S. Heo²;¹CHA University, Seong-Nam Si, Korea, ²CHA University, SungNam, Korea

ELK3 is a member of the Ets family of transcription factors. Its expression is associated with angiogenesis, vasculogenesis and chondrogenesis. ELK3 inhibits endothelial migration and tube formation through the regulation of MT1-MMP transcription. This study assessed the function of ELK3 in breast cancer (BC) cells by comparing its expression between basal and luminal cells in silico and in vitro. In silico analysis showed that ELK3 expression was higher in the more aggressive basal BC cells than in luminal BC cells. Similarly, in vitro analysis showed that ELK3 mRNA and protein expression was higher in basal BC cells than in normal cells and luminal BC cells. To investigate whether ELK3 regulates basal cell migration or invasion, knockdown was achieved by siRNA in the basal BC cell line MDA-MB-231. Inhibition of ELK3 expression decreased cell migration and invasion and downregulated MT1-MMP, the expression of which is positively correlated with tumor cell invasion. In silico analysis revealed that ELK3 expression was associated with that of MT1-MMP in several BC cell lines (0.98 Pearson correlation coefficient). Though MT1-MMP expression was upregulated upon ELK3 nuclear translocation, ELK3 did not directly bind to the 1.3 kb promoter region of the MT1-MMP gene. These results suggest that ELK3 plays a positive role in the metastasis of BC cells by indirectly regulating MT1-MMP expression.

P1887**Board Number: B394****Cytochrome c Oxidase dysfunction induces retrograde signaling and invasive phenotype.**S. Srinivasan¹, M. Guha², D. Dong¹, G. Ruthel¹, K. Whelan¹, Y. Uchikado³, S. Natsugoe³, H. Nakagawa¹, N.G. Avadhani⁴;¹University of Pennsylvania, Philadelphia, United States, ²Animal Biology, University of Pennsylvania, Philadelphia, PA, ³Kagoshima University, Kagoshima, Japan, ⁴Department of Animal Biology School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA

Defects in OXPHOS complexes are associated with several diseases including cancer. Mitochondrial DNA (mtDNA) defects such as deletions, point mutations and reduced DNA copy number, that affect activities of electron transport chain (ETC) complexes strongly correlate with incidence of cancer. Cytochrome oxidase (CcO) is the terminal enzyme of the ETC that catalyzes the transfer of electrons from reduced cytochrome C to oxygen. The mammalian enzyme is a bigenomic complex with three subunits coded by MtDNA and ten by nuclear DNA. MtDNA mutations affecting CcO subunits I, II and III are prevalent in prostate, pancreatic, colon and ovarian cancers. These mutations cause premature chain termination,

impaired proton pumping or reduced kinetic activity and possibly due to severe structural perturbation. All these mutations result in significant loss of enzyme activity and respiratory defect. However studies on loss of function of nuclear subunits affecting Cytochrome oxidase activity related to tumor progression are limited. Earlier work from our lab showed that various conditions like exposure to long term hypoxia, ischemia-reperfusion and treatment with ethanol result in selective degradation of nuclear subunits IVi1 and Vb of CcO leading to a loss of activity. Here we show a role for the disruption of CcO in cancer progression. Genetic silencing of the CcO complex in C2C12 myoblasts and loss of activity resulted in metabolic shift to glycolysis. This was complemented by increased expression of glucose transporters and glucose uptake. Interestingly, these cells formed multicellular colonies on soft agar plates suggesting anchorage independent growth, a property absent in normal cells. Disruption of CcO complex caused loss of transmembrane potential and induction of Ca²⁺/Calcineurin mediated retrograde signaling. Whole genome expression analysis showed significant up regulation of genes involved in cell signaling, extracellular matrix interactions, cell morphogenesis, cell motility and migration. In support of these, esophageal tumors from human patients revealed reduced CcO subunits IVi1 and Vb. Our results show that mitochondrial ETC defect initiates a retrograde signaling, which induces a tumorigenic phenotype. Supported by NIH grants CA-22762 and GM-34883.

P1888

Board Number: B395

Mitochondrial retrograde signaling induces epithelial-mesenchymal transition and generates breast cancer stem cells.

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Metastatic breast tumors undergo epithelial-to-mesenchymal transition (EMT) which renders them resistant to therapies targeted to the primary cancers. The mechanistic link between MtDNA reduction, often seen in breast cancer patients and EMT is unknown. We demonstrate that reducing MtDNA content in human mammary epithelial cells (hMECs) activates Calcineurin-dependent mitochondrial retrograde signaling pathway which induces EMT-like reprogramming to fibroblastic morphology, loss of cell polarity, contact inhibition and acquired migratory and invasive phenotype. Notably, MtDNA-reduction and mitochondrial respiratory stress in hMECs correlates with a high percentage of CD44^{hi}/CD24^{lo} cells, which in serial dilution assays showed increased self-renewal and mammosphere-forming capacity in 3D culture, characteristics of breast cancer stem cells. Mechanistically, we showed that Mt-retrograde signaling in hMECs regulates alternative splicing (AS) by loss of ESRP1, which favors

EMT. In addition to retrograde signaling markers, there is an induction of mesenchymal genes but loss of epithelial markers in these cells. The changes are reversed by either restoring the MtDNA content or knockdown of calcineurin α mRNA indicating the causal role of retrograde signaling in EMT. Our results point to a new therapeutic strategy for metastatic breast cancers targeted to the mitochondrial retrograde signaling pathway for abrogating EMT and attenuating cancer stem cells, which evade conventional therapies. We report a novel regulatory mechanism by which low MtDNA content generates EMT and cancer stem cells in hMECs. (Supported by NIH grant CA-22762 to NGA).

P1889

Board Number: B400

Is HPV causative factor in oral cancer?.

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Human papillomavirus (HPV) is the main source of cervical cancer. Recently, HPV, associated with oropharyngeal cancer has been reported in some literatures, but there are a few affordable data about incidence of HPV in oral cancer. The purpose of this study is to review the previous articles and to find the incidence and types of HPV in specific areas of oral cavity. We examined total 239 patients for HPV detection. Specimens were extracted from main mass during surgery, and performed for HPV typing by DNA chip kit. Available DNA chip kit (MY-HPV chip kit[®], Mygene Co., Korea) was used for detection of low risk HPV types (6, 11, 34, 40, 42, 43, 44) and high risk HPV types (16, 18, 31, 33, 35, 39, 45, 51, 52, 54, 56, 58). DNA isolation was performed using DNA extraction buffer via centrifugation, and DNA amplification was carried on by polymerase chain reaction (PCR) using primer, B-globin primer for control group, taq polymerase, template DNA, four types of deoxynucleotides (dNTP) and buffer solution. Second PCR product was mixed with hybridization buffer for hybridization. Among 239 patients, histologic results showed that most of them were squamous cell carcinomas, 174 patients. Among the thirteen HPV positive cases, twelve cases was squamous cell carcinoma (6.90%). In SCC patients, five cases on tongue (11.9%), six cases on lower gum (10.5%), one case on upper gum (2.8%) had positive results. Among thirteen HPV positives, the number of high risk types was seven. Because prognosis of HPV positive tumor is known to be much better than that of HPV negative tumor, HPV detecting methods, such as HPV DNA chip method, may be great values to use. By evaluating the incidence of HPV in oral cancer, we can predict the prognosis of oral cancer patients and treat the patient properly. Vaccine may also be used to prevent HPV induced carcinogenesis in patients who are prone to develop oral cancer. "This research was supported by the International Research & Development Program of the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Plannig(Grant number: 2014K1A3A9A01033785)"

P1890**Board Number: B401****CXCR4 Gradient Sensing generates unique signaling kinetic signatures, and drives ERK to Invadopodia in Metastatic Breast Cancer Cells.**B. Winant¹, J. Freed¹, J. Stewart¹, M. Sobel¹, M. Campbell¹, K.A. Myers², C.C. Moore¹;¹Department of Pharmaceutical Sciences, Philadelphia College of Pharmacy, University of the Sciences, Philadelphia, PA, ²Department of Biological Sciences, Misher College of Arts and Sciences, University of the Sciences, Philadelphia, PA

During development, the chemokine G protein-coupled receptor CXCR4 is essential for directional migration of select neuronal, cardiovascular, and hematopoietic cells in response to sensing a gradient of SDF ligand. During disease, the SDF-CXCR4 axis plays a critical role in breast cancer metastasis by promoting the directional migration of primary tumor cells to distal SDF-expressing organs. Despite these observations, the biochemical signaling pathways downstream of motility inducing SDF gradients are unknown. Previously, we identified unique CXCR4 pathways and signaling kinetic signatures associated with SDF gradient sensing, including a novel Arf6-ERK motility pathway and a sustained MAPK signaling pathway atypical for GPCRs but reminiscent of GFR signaling during malignancy. Here we extended these findings using microarray and IB analysis, and identified that gradient SDF also leads to sustained Akt activation, and both the Akt and ERK pathways are inhibited by wortmannin or LY294002. Notably, both pathways regulate processes critical to the malignant phenotype including invadopodia formation. Here we also extended our previous findings using Matrigel invasion assays, pseudopodia isolation, and confocal imaging of invadopodia, and determined that the novel Arf6-ERK motility pathway also mediates cell invasion in metastatic breast cancer cells, and that Arf6 drives ERK to membranous structures including pseudopodia and invadopodia in response to gradient SDF. These results provide insight into the biochemical signaling pathways downstream of CXCR4 gradient sensing, with implications on the invasive phenotype of metastatic breast cancer cells.

These studies were supported by NIH grant GM-097718. JS, MS, MC contributed equally.

P1891**Board Number: B402****A noncanonical Frizzled2 pathway regulates epithelial-mesenchymal transition, tumor progression, and metastasis.**T. Gujral¹, M. Chan¹, L. Peshkin^{1,2}, P.K. Sorger^{1,3}, M.W. Kirschner^{1,2}, G. MacBeath¹;¹Systems Biology, Harvard Medical School, Boston, MA, ²Department of Systems Biology, Harvard Medical School, Boston, MA, ³Harvard Med Sch, Boston, MA

Wnt signaling plays a critical role in embryonic development, and genetic aberrations in this network have been broadly implicated in colorectal cancer. Less is known, however, about its role in epithelial-mesenchymal transition (EMT) and cancer metastasis. We found that the Wnt receptor Frizzled2 (Fzd2)

and its ligands Wnt5a/b are elevated in metastatic liver, lung, colon, and breast cancer cell lines and in high-grade tumors, and that their expression correlates with markers of EMT. Pharmacologic and genetic perturbations revealed that Fzd2 drives EMT and cell migration through a previously unrecognized, non-canonical pathway that includes Fyn and Stat3; a gene signature regulated by this pathway predicts metastasis and overall survival in patients. We have developed an antibody to Fzd2 that reduces cell migration and invasion and inhibits tumor growth and metastasis in xenografts. We posit that targeting this pathway could provide benefit for patients with tumors expressing high levels of Fzd2.

P1892

Board Number: B403

THE ROLE OF SIALOPHORIN (CD43) DURING TUMOR PROGRESION.

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Mucins constitute a selective molecular barrier at the epithelial surface. Alterations in their expression or glycosylation profile impact cellular growth, differentiation, adhesion, transformation and invasion, promoting the development of cancer. **CD43** (Sialophorin) is a mucin expressed by lymphoid cells. However, CD43 expression has been documented in several types of non-lymphoid tumors, such as lung, colon and breast cancer, correlating with poor prognosis. We have shown that CD43 promotes cell transformation by abrogating the contact inhibition of growth through a molecular mechanism involving Akt-dependent Merlin phosphorylation and degradation, that eventually lead to cell survival, proliferation and tumor growth in the CD43+ A549 and Caski cells. The aim of this study is to explore the biological effects and mechanisms of CD43 expression confers to highly invasive breast cancer cells **MDA-MB-231** lacking the expression of endogenous CD43. Through a structure/function analysis, we will discuss how the expression of three different constructs of the human CD43 molecule (CD43 WT, CD43 Δ Pro (-25 a.a. from COOH-terminal), and CD43 Δ IC (a form devoid of the intracellular domain) impacts processes such as proliferation, survival, migration, and invasion of MDA-MB-231 tumor cells. Likewise, we will analyze how the signaling pathways related to these biological processes, among which the PI3K/Akt and Wnt/ β -catenin pathways (involved in migration and invasion) are modified by CD43 expression. Our results provide clues about the importance of CD43 in the breast cancer biology. (Partially funded by CONACyT and PAPIIT/UNAM, Mexico).

Tumor Microenvironment

P1893

Board Number: B404

Endothelial podosome rosettes regulate vascular branching in tumor angiogenesis.

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The mechanism, by which angiogenic endothelial cells break the physical barrier of vascular basement membrane and consequently sprout, forming new vessels in mature tissues, is unclear. Here, we show that angiogenic endothelium is characterized by the presence of functional podosome rosettes. These extracellular matrix-degrading and adhesive structures are precursor of de novo branching points and represent a key event in the formation of new blood vessels. VEGF-A stimulation induces the formation of endothelial podosome rosettes by up-regulating integrin $\alpha 6\beta 1$; in contrast, the binding of $\alpha 6\beta 1$ integrin to vascular basement membrane laminin impairs the formation of podosome rosettes by restricting $\alpha 6\beta 1$ integrin to focal adhesions and hampering its translocation to podosomes. Using ex vivo sprouting angiogenesis assay, transgenic and knock-out mouse models and human tumor samples analysis, we provide the first evidence that endothelial podosome rosettes control blood vessels branching and are critical regulators of pathological angiogenesis.

P1894

Board Number: B405

Impact of mechanical stress on Multicellular Tumor Spheroids proliferation.

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A tumor micro-region consists of a 3D heterogeneous cell population in which cancer cells growth is influenced by interaction with the microenvironment. The crosstalk between tumor cells and microenvironmental components, including the extracellular matrix (ECM), fibroblasts, endothelial and immune cells, is essential for tumor progression and plays a key role in drug resistance. In such a complex environment, solid tumors are also subjected to mechanical stress that influences their growth rate and development. Indeed, modification in mechanical homeostasis within tissues are also observed during tumor growth. However, little is known about the effects of such mechanical stress on tumor cell

biology and their pharmacological consequences. To explore this issue, we investigated the impact of mechanical stress on cell proliferation in Multicellular tumor spheroids (MCTS) in which cancer cells are cultured as 3D organized spheres. Such complex multicellular model reproduces the cell-cell and cell-matrix interactions found in solid tumors. Moreover, MCTS can grow up to several hundred micrometers in diameter, thus progressively displaying a gradient of proliferating cells similar to what is found in tumor micro-regions. Specifically, in large spheroids, dividing cells are located in the outmost layers while quiescent cells are found in more central hypoxic and nutrient-poor regions. Using this model, we showed, by using PDMS microdevices, that spheroids can grow under mechanical confinement and that reciprocal resistance from the PDMS walls opposed to spheroids growth induces mechanical stress. In these conditions, we demonstrated that growth-induced mechanical stress induces mitotic accumulation inside spheroids. We then monitored the impact of mechanical stress on the progression in mitosis using light sheet fluorescence microscopy. We developed specific sample holders that enabled us to explore 3D mitosis dynamics inside spheroids in control and confined conditions. By measuring the duration of each phase of mitosis, we demonstrated that mechanical stress induces a prometaphase delay. As growing solid tumors also exert forces on their microenvironment, in order to evaluate the mechanical properties of growing spheroids, we designed and produced microdevices arrays of high aspect ratio pillars. Using these microdevices, we investigated the forces exerted by spheroids from various cell lines. We will present our latest results on the impact of these specific controlled environments on cell proliferation and 3D organization of spheroids.

- Aoun L, Weiss P, Laborde A, Ducommun B, Lobjois V, Vieu C. *Lab Chip*. 2014 Jun 2;14(13):2344-53. *: co-corresponding authors
- Desmason A, Frongia C, Grenier K, Ducommun B, Lobjois V. *PLoS One*. 2013 Dec 3;8(12):

P1895

Board Number: B406

Retention of Somatic Mutations in Cancers by Gain in pH Sensing.

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A critical unresolved question in cancer is how somatic mutations are selectively retained and what selective pressures direct this process. One common feature of cancers is constitutively increased intracellular pH (pHi) that enables diverse cancer cell behaviors, including increased cell proliferation and migration as well as metabolic adaptation and decreased apoptosis. We propose that increased pHi provides a selective pressure for the retention of somatic mutations in some human cancers and that these mutations provide an adaptive advantage to the increased pHi of cancer cells. Here, we focus on Arg>His mutations, predicting that replacing a non-titratable arginine (pKa 12) with a titratable histidine (pKa 6.5) may confer pH sensitive function (pH sensing) to the mutant protein. To determine whether Arg>His mutations are selectively retained, we performed bioinformatics analyses of COSMIC and

tumor/normal paired sequencing datasets. These analyses revealed that Arg>His mutations are significantly overrepresented in a subset of cancers including stomach, prostate, colorectal, and glioma. Arg>His mutations are enriched even after accounting for codon bias and CpG site frequency, suggesting selective pressure may drive retention. Next, we investigated whether Arg>His mutations confer an adaptive advantage to cancer cells. We identified Arg>His somatic cancer mutations in p53 and EGFR and tested mutants for a gain in pH sensing. First, we show that the most highly recurrent p53 mutation in cancer, p53-R273H, has pH-sensitive promoter binding with decreased DNA binding at the increased pHi of cancer cells, while wild-type p53 promoter binding is pH insensitive. We predict that Arg273 interacts with the phosphate backbone of DNA, and His273 is less able to form stable interactions with negatively charged DNA at higher pHi. Second, we investigated two Arg>His mutations in EGFR that are found in mesotheliomas and lung cancer: R776H and R831H. Using both in vitro and cell-based assays, we found that the kinase activity of these mutants is pH sensitive with increased activity at higher pH, while wild-type EGFR kinase activity is pH insensitive. Additionally, molecular dynamics simulations suggest potential mechanisms for pH sensing by EGFR-R776H, including critical conformational changes in the α C helix of the kinase domain resulting from changes in histidine protonation. These data suggest that Arg>His somatic mutations can confer adaptive pH sensing to p53 and EGFR. Together, our studies integrate whole-genome bioinformatics, biochemical, cellular, and structural analyses to show that the increased pHi of cancer cells may be a selective pressure driving retention of Arg>His mutations that may confer adaptive pH sensing to tumorigenic proteins.

P1896

Board Number: B407

PDLIM2 is required for macrophage subset differentiation with implications for cytokine production in tumor microenvironment.

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The PDZ-LIM domain protein PDLIM2 is associated with an invasive cancer phenotype [1] and integrates cytoskeleton signalling with regulation of gene expression during epithelial cell differentiation [2]. PDLIM2 is also expressed in hematopoietic cells and regulates NF κ B and STAT transcription factor stability in macrophages and lymphocytes [3] [4]. Macrophage infiltration may correlate with a poor prognosis in many types of human cancer. We hypothesized that PDLIM2 activity in macrophages may influence their phenotype and thereby the cancer microenvironment. To test this we investigated PDLIM2's contribution to the classically activated pro-inflammatory subtype (M1) and the alternatively activated (M2) macrophage subtypes using bone marrow derived macrophages (BMDM) from PDLIM2 WT and knockout (-/-) mice that were stimulated in vitro. We found that PDLIM2 moves from the nucleus to the cytoplasm in response to different activation stimuli, although NO and iNOS levels were similar in BMDM from WT and -/- mice. BMDM from PDLIM2 -/- mice exhibited altered inflammatory cytokine profiles with increased IL-6 and decreased IL-1 β compared to WT BMDM. MIF expression and nuclear retention of the IRF-3/CPB complex was also enhanced in PDLIM2-/- macrophages. M2-polarized

macrophages derived from PDLIM2^{-/-} BMDM exhibited reduced migratory capacity, delayed pro-angiogenic cytokine production, and failed to suppress pro-inflammatory cytokine expression. Altogether, our results demonstrate that PDLIM2 is essential for complete M2 polarization and for suppression of the M1 phenotype. Thus PDLIM2 expression in macrophages could have impact cancer progression by controlling macrophage phenotype and cytokines in the tumour microenvironment.

References: [1] Gene expression profiles in cells transformed by overexpression of the IGF-I receptor. Loughran G, Huigsloot M, Kiely PA, Smith LM, Floyd S, Ayllon V, O'Connor R. *Oncogene*. 2005 Sep 8;24(40):6185-93. [2] PDLIM2 regulates transcription factor activity in epithelial-to-mesenchymal transition via the COP9 signalosome. Bowe RA, Cox OT, Ayllón V, Tresse E, Healy NC, Edmunds SJ, Huigsloot M, O'Connor R. *Mol Biol Cell*. 2014 Jan;25(1):184-9. [3] Sequestration of PDLIM2 in the cytoplasm of monocytic/macrophage cells is associated with adhesion and increased nuclear activity of NF-kappaB. Healy NC, O'Connor R. *J Leukoc Biol*. 2009 Mar;85(3):481-90. [4] SLIM is a nuclear ubiquitin E3 ligase that negatively regulates STAT signaling. Tanaka T, Soriano MA, Grusby MJ. *Immunity*. 2005 Jun;22(6):729-36.

P1897

Board Number: B408

Tumor cell motility in microenvironment context: From intravital microscopy to systems view.

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While it has been established that a number of microenvironment components affect the likelihood of metastasis, the influence of various determinants between microenvironment and tumor cell phenotypes is poorly understood. Here we have examined factors affecting microenvironment control over two different tumor cell motility phenotypes required for metastasis. By high-resolution multiphoton microscopy of mammary carcinoma in mice, we detected two phenotypes of motile tumor cells, different in locomotion speed. Slow- and fast-locomotion occurred at different spatio-temporal coordinates and only 3/184 regions showed both behaviors. Slower tumor cells exhibited invadopodia, small, cortactin-rich protrusions with capability of degrading extracellular matrix. To understand how the tumor microenvironment controls invadopodium formation and tumor cell locomotion in general, we systematically analyzed components of the microenvironment previously associated with cell invasion and migration. No single microenvironmental property was able to predict the locations of tumor cell phenotypes in the tumor when used in isolation or combined linearly. To solve this, we combined multiphoton microscopy with the Support Vector Machine (SVM) algorithm to classify phenotypes in a nonlinear fashion. This approach identified conditions that promoted either motility phenotype. We then demonstrated that varying one of the conditions can either change the number of tumor cells with invadopodia or switch tumor cell behavior in a context-dependent manner. In addition,

to establish the link between motility phenotypes and cell fates, we photoconverted and monitored the fate of tumor cells in different microenvironments, finding that only tumor cells in the invadopodium-rich microenvironments, but not in microenvironments where fast-locomotion occurred, have degraded surrounding extracellular matrix and disseminated. The number of invadopodia positively correlated with ECM degradation, while the MMP-inhibitor inhibiting metalloproteases inhibited eliminated degradation and stopped lung metastasis, consistent with a direct link among invadopodia, ECM degradation and metastasis. In summary, we have detected and characterized two phenotypes of motile tumor cells in vivo, which occurred in spatially distinct microenvironments of primary tumors. We show how machine-learning analysis can classify heterogeneous microenvironments in vivo to enable prediction of motility phenotypes and tumor cell fate. The ability to predict the locations of tumor cell behavior leading to metastasis in mouse models of breast cancer models has the potential for identifying may lead towards understanding the mechanisms behind the heterogeneity of response to treatment.

P1898

Board Number: B409

Developmental control of regeneration signaling determines the timing and location of neoplastic transformation in epithelial tumors.

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The emergence of several tumor types in humans is correlated with specific developmental transitions (e.g. thyroid carcinoma and malignant melanoma at puberty). Since the expression of tumorigenic phenotypes by cells depends on the interaction between genetic changes within the cells and their environment, tumor formation in these instances may rely on developmental signals produced within specific tissues at specific times.

To examine how developmental signaling context interacts with cellular mutations to produce tumorigenic phenotypes, we are studying the emergence of neoplastic epithelial tumors that result from mutation in the tumor-suppressor genes *avalanche* or *lethal giant larvae* in *Drosophila* wing imaginal discs. We have observed that the expression of the neoplastic tumor phenotype resulting from either of these mutations begins at a specific time in development that is correlated with the increased activity of a steroid endocrine signal, ecdysone. We have shown that ecdysone promotes neoplastic transformation in *avalanche* or *lethal giant larvae* mutant cells by signaling through its nuclear receptor (EcR) within mutant cells. Furthermore, we demonstrate that the role of ecdysone in promoting tumor progression is through regulating transcription of a specific gene, *wingless*, that functions in normal development of the wing imaginal disc, and is activated in both neoplastic tumors and during epithelial tissue regeneration. The ecdysone-dependent activation of *wingless* in both neoplastic tumors and during regeneration function through a similar mechanism and gene regulatory region. Furthermore, activation of a regenerative response in wing discs through damage can potentiate tumorigenesis. Thus,

this regulation of *wingless* provides a direct link between tissue regenerative signaling and tumor development. Additionally, in undamaged wing imaginal discs we observe a regional bias for tumor initiation in *lethal giant larvae* mutant clones. We propose that this bias reflects underlying differences in regenerative capacity across the tissue.

In summary, we observe that neoplastic transformation by tumor-suppressor mutations is dependent on a developmentally-regulated regeneration signaling response. The capacity of the tissue to activate this regenerative pathway determines the timing and location of tumor formation.

P1899

Board Number: B410

Induction of Carcinoma-Associated Fibroblasts in 3D Cell Coculture.

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Carcinoma-associated fibroblasts (CAFs) play a critical role in tumor progression and are associated with poor prognoses, high-grade malignancy, and hindrance of anti-cancer drug therapies. However the process in which normal fibroblasts (NFs) are transformed into CAFs is yet to be elucidated. To gain insight of this process, we constructed a coculture system where GFP-labeled NIH3T3 fibroblasts were cocultured in 3D extracellular matrix (ECM) with preformed acini containing normal, neoplastic (early stage) or metastatic (late stage) cancer mammary gland epithelial cells, respectively. The dynamic interaction between fibroblasts and epithelial cells were recorded by timelapse microscopy between 6-hour and 20-hour post seeding and tracked to characterize the behavioral difference of the cocultures. It was found that both neoplastic and metastatic cancer cells generated at least 2-fold more mechanical forces to remodel the ECM organization compared to the normal epithelial cells. Furthermore fibroblasts cocultured with cancer epithelial cells exhibited faster mobility compared to normal controls. Immunoblots revealed CAF markers such as phospho-myosin and vimentin are elevated in fibroblasts cocultured with neoplastic and metastatic epithelial cells, indicating CAF transformation can be initiated within 20 hours after NFs are in contact with cancer cells.

P1900

Board Number: B411

Morphine and Mast Cell Activation Stimulate Endothelial Dysfunction.

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Although morphine is currently a predominant treatment for cancer-induced chronic pain, its pharmacological effects accelerate tumor progression. Morphine stimulates mast cells in the skin and

tumors of mice, leading to the release of cytokines and neuropeptides. Additionally, morphine-induced activation of mast cells stimulates angiogenesis, tumor progression, and contributes to vascular leakage leading to neurogenic inflammation and pain. Therefore, we examined if mast cell activation, particularly the release of cytokines and neuropeptides, has direct effects on tumor endothelium. To demonstrate the developmental spectrum of human breast carcinoma, transgenic mice with a rat C3(1) simian virus 40 large tumor antigen fusion gene were used. We also used primary mouse brain microvascular endothelial cells (MBMEC). We observed that morphine alters the tumor microenvironment by stimulating inflammation and neuroinflammation. IL-17, the master cytokine mediating the release of other neuropeptides and cytokines, was significantly increased in tumors of morphine treated mice vs PBS treatment (p

P1901

Board Number: B412

Extracellular vesicles from women with breast cancer promotes migration in MDA-MB-231 breast cancer cells.

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Breast cancer is the most common cancer and the leading cause of death in women worldwide and affects countries at all levels of development. Extracellular vesicles (EVs) are small membrane-enclosed sacs of endosomal and plasma membrane origin secreted by normal and malignant cells. The EVs represent an important mode of intercellular communication by serving as vehicles for transfer between cells of membrane and cytosolic proteins, lipids, and RNA. Particularly, EVs from tumor cells mediate many stages of tumor progression including angiogenesis, escape from immune surveillance, invasion and metastasis. The aim of this work was to determine the EVs number in plasma of breast cancer patients and healthy women, and whether EVs from breast cancer patients are able to induce migration of MDA-MB-231 breast cancer cells. We analyzed plasma fractions enriched in EVs and deprived of platelet-derived EVs obtained by differential centrifugation from blood samples of twenty-one Mexican female patients (median age 54 years, range 38-80 years) with biopsy-proven of breast cancer at different clinical stages and without receiving therapy. The control group consisted of 13 healthy females (median age 42.7 years, range 16-86 years). EVs number was evaluated by BD TruCOUNT Tubes (BD Biosciences) and migration was studied by scratch-wound assay. Our findings demonstrated that EVs number is higher in women with breast cancer with stages II and III compared with control group. In addition, EVs from breast cancer patients are able to induce migration of MDA-MB-231 cells and secretion of MMP-9.

P1902**Board Number: B413****Extracellular vesicles from MDA-MB-231 cells stimulated with linoleic acid induce an epithelial to mesenchymal transition-like process in mammary epithelial cells MCF10A.**O. Galindo-Hernandez¹, C. Rodriguez-Monterrosas¹, N. Serna-Marquez¹, R. Castillo-Sanchez¹, P. Cortes-Reynosa¹, E.P. Salazar¹;¹Biologia Celular, CINVESTAV, Mexico City, Mexico

Extracellular vesicles (EVs) are fragments of the plasma membrane secreted from cytoplasmic membrane compartments by normal and malignant cells and their function appear to be dependent on cargo they carry and the cell type which they originate. It has been described that EVs mediate many stages of tumor progression including angiogenesis, escape from immune surveillance and extracellular matrix degradation. Linoleic acid (LA) is an essential polyunsaturated fatty acid that induces migration and invasion in breast cancer cells. However, the role of EVs secreted from MDA-MB-231 cells stimulated with LA as promoter of the epithelial-mesenchymal-transition (EMT) process in mammary non-tumorigenic epithelial cells MCF10A remains to be studied. In the present study, we demonstrate that treatment of MDA-MB-231 cells with 90 μ M LA for 48 h does not induce an increase in the number of secreted EVs, however all the secreted EVs are taken up for MCF10A cells. In contrast, only the EVs isolated from supernatants of MDA-MB-231 stimulated with 90 μ M LA for 48 h are able to induce a transient down-regulation of E-cadherin expression, an increase of vimentin and N-cadherin protein levels, as well as an increase of MMP-2 and -9 secretions in MCF10A cells. Furthermore, EVs from MDA-MB-231 cells stimulated with LA also induce an increase of NF κ B-DNA binding activity, migration and invasion. In summary, our findings demonstrate, for the first time, that EVs isolated from supernatants of MDA-MB-231 stimulated with 90 μ M LA for 48 h induce an EMT-like process in human mammary non-tumorigenic epithelial cells MCF10A.

P1903**Board Number: B414****PROTEASE ADAMTS-1 ROLE IN LOCAL AND SYSTEMIC INVASION OF FIBROSARCOMA CELLS.**V.M. Freitas¹, M.C. Cruz², H.G. NORIEGA³;¹Department of Cell and Developmental Biology, University of Sao Paulo, Institute of Biomedical Sciences, Sao Paulo SP, Brazil, ²Center of Facilities and Support Research, University of Sao Paulo, Institute of Biomedical Sciences, Sao Paulo, Brazil, ³Departament of Cell and Developmental Biology, University of Sao Paulo, Institute of Biomedical Sciences, São Paulo, Brazil

Fibrosarcoma is a malignant tumor with the presence of immature proliferating fibroblast. The growth and malignancy of a tumor is dictated by the surrounding microenvironment. The extracellular matrix is

a reservoir of cell binding proteins and growth factors. ADAMTS-1 (*a disintegrin and metalloproteinase with thrombospondin motifs*) is a secreted protease that modified the extracellular matrix during malignant progression. We aim to evaluate the role of ADAMTS-1 within the tumor microenvironment of fibrosarcoma. Furthermore, we study the effects of the growth factors-binding ADAMTS-1. For *in vivo* studies we used zebrafish embryos at 48 hours post fertilization. HT1080 fibrosarcomacells (dyed in red) were resuspended in medium with 5nM ADAMTS-1 and injected into embryos's heart. Confocal microscopy images showed that in presence of ADAMTS-1, tumor cells were into the vasculature and some of them were arrested inside the intra segmental vases. Also a higher number of tumor cells interacted with the surrounding tissue and disseminate to distant sites. Moreover, to examine the metastatic behavior of fibrosarcoma cells, we injected them into the perivitelline space. We observed tumor cells were restricted into the yolk sac with invadopodia formation in presence of ADAMTS-1. For *in vitro* studies, Bromodeoxyuridine (BrdU) incorporation assay was used to evaluate cell proliferation. According to expected, increasing concentrations of VEGF led to increase of HT1080 cell proliferation. To analyze the effect of ADAMTS-1 overexpression in HT1080 cell migration, we used time-lapse imaging and MTrackJ plugin. We observed that ADAMTS-1 overexpression not stimulated the migratory activity of fibrosarcoma cells, and these cells presented a mean velocity of 4.20 $\mu\text{m}/\text{hour}$, whereas the velocity of control cells was 18.73 $\mu\text{m}/\text{hour}$. In contrast, the treatment of cells with ADAMTS-1 overexpressed with 10ng/ml VEGF leads to increase of cell migration with a mean velocity of 26.74 $\mu\text{m}/\text{hour}$, whereas the velocity of the control cells treated with VEGF was 7.23 $\mu\text{m}/\text{hour}$. VEGF was probably involved in ADAMTS-1 effects on fibrosarcoma cells. Our results suggest that ADAMTS-1 induced the interaction of fibrosarcoma cells with the stroma *in vivo*. Also ADAMTS-1 reduced fibrosarcoma cells migration *in vitro*. This study was approved by the Ethics Committee on Animal Use - CEUA (ICB-USP) n^o009 pg. 132. Supported by The State of São Paulo Research Foundation (FAPESP grants 2010/07966-1, 2012/24108-2).

P1904

Board Number: B415

Exchange of microvesicles between breast tumor cells and normal fibroblasts.

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The successful development of tumors is driven by the tissue microenvironment. Cancer development is not only dependent on mutation in cancer cells, but it is also dependent on interaction of cells and their surrounding stroma. Some cell types release vesicular structures into the extracellular space. These vesicles would be involved in cellular signaling and tumor progression. The aim of this study was to analyze *in vitro* interactions between cells of tissue microenvironment (fibroblasts) and tumor cells. Co-

culture assays were carried out with non-tumoral fibroblasts and tumor breast cells (MDA-MB-231). Cells were loaded with different vital dyes. Tumor cells were plated on top of a fibroblasts monolayer. After 24 hours cells were fixed and analyzed by confocal microscopy. Three-dimensional reconstruction showed exchange of vesicles with heterogeneous size. In order to characterize these vesicles, cells were analyzed by transmission electron microscopy (TEM). Conditioned medium from tumor cells was centrifuged, and vesicles counted and analyzed by NanoSight. The conditioned medium was further characterized by LC-MS/MS and immunoblot. Protein AHNAK (desmoyokin) was identified in conditioned medium by mass spectrometry. Immunofluorescence showed the presence of the AHNAK in the exchanged vesicles from co-cultured cells. Co-culture and NanoSight showed high number of vesicles in tumor cells compared to normal cells. Our results showed evident microvesicle exchange between breast cancer cells and stroma. The protein AHNAK was detected in exchanged vesicles, and could represent a microenvironmental molecule influencing mammary tumor biology. This study was approved by and Ethics Committee on Human Research - CEPISH (ICB-USP). Supported by FAPESP (2010/07699-1 and 2011/09472-7).

P1905

Board Number: B416

Hypoxic primary tumor stress microenvironments prime DTCs in lungs for dormancy.

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The mechanisms behind dormancy of disseminated tumor cells (DTCs) are unclear. Further, whether primary tumor microenvironments might influence DTC fate has never been explored in situ. We found that breast tumors enriched for a specific dormancy signature (DS+) displayed longer metastasis-free periods than those poor (DS-) for the signature. Key genes in the DS induce quiescence and are also regulated by hypoxia. Interestingly, a main response of tumor cells to hypoxia is growth arrest. We hypothesized that hypoxic primary tumor microenvironments may spawn a subpopulation of DTCs that, by virtue of becoming dormant, might escape therapies and eventually fuel incurable metastasis. We used H2B-EGFP inducible HEp3 HNSCC and photo-switchable (green-to-red fluorescence) H2B-Dendra2 expressing MDA-MB-231 and ZR-75-1 human breast cancer cell lines to identify cells from hypoxic microenvironments. To initiate spatially defined hypoxic microenvironments in primary tumors we implanted induction NANO IntraVital Devices (iNANIVIDs) carrying hypoxia-mimetic agents (i.e. desferrioxamine - DFOM) in T-HEp3 tumors in vivo (chicken chorioallantoic membrane (CAM) model) or

exposed cultured MDA-MB-231 or ZR-75-1 cells in vitro to either 21% or 1% O₂. The regions influenced by the DFOM-iNANIVID displayed upregulation of p27, NR2F1 and DEC2 (dormancy genes), as well as induction of hypoxia markers (GLUT1, HIF1 α). We found that lung DTCs derived by mouse tail vein injection of hypoxia induced H2B-EGFP T-HEp3 or H2B-Dendra2 MDA-MB-231 cells were primed to enter dormancy in lungs > 2 weeks after extravasation, as measured using H2B-EGFP and H2B-Dendra2-RED label retention. Using Vimentin to screen for HEp3 tumor cells in lungs, we found that DTCs originating from the iNANIVID induced hypoxic regions showed a dormant profile as evidenced by significant upregulation of p27, NR2F1 and DEC2 compared to DTCs originating from a normoxic milieu. Further, analysis in 3D culture models revealed that ER+/DS+ breast cancer cells (ZR-75-1) are more prone to enter a prolonged quiescent state after a brief exposure to hypoxia (1% O₂) while this response is not observed in triple negative/DS- breast cancer cells (MDA-MB-231). We propose that hypoxic primary tumor stress microenvironments induce a subpopulation of tumor cells to express the DS. Upon spreading these DTCs may be more prone to enter dormancy, evade anti-proliferative therapies and eventually fuel metastasis.

P1906

Board Number: B417

Matrix Compliance Regulates Tetraploidy in Mammary Epithelial Cells.

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Introduction: Cancer remains the second leading cause of death worldwide. The research that drove most current cancer therapies focused primarily on cancer cells themselves, when in reality tumor cells are constantly communicating with the local microenvironment. Through defining features such as hypoxia and stiffness, the tumor microenvironment can cause changes in molecular signaling that result in tumorigenic phenotypes. This study aims to elucidate whether the mechanical rigidity of the substratum regulates chromosomal instability in the form of tetraploidy. Tumors are inherently stiffer than normal tissue, and stiffness has been shown to cause changes in cell growth and proliferation. Similarly, cell cycle errors have long been linked to chromosomal abnormalities, a phenotype common to 85% of solid cancers. The mechanisms by which tissue compliance regulates chromosomal instability remain unclear, but may provide an avenue for new cancer treatments. Here, we investigated the effects of substratum stiffness on tetraploidy.

Materials and Methods: Mammary epithelial cells were plated on polyacrylamide gels of varying compliance (to mimic normal and tumor tissue) conjugated with the extracellular matrix protein fibronectin. Using epifluorescent microscopy, tetraploidy was easily observed as multinucleation. The signaling pathways controlling mechanical regulation of multinucleation were also investigated.

Results and Discussion: Mammary epithelial cells cultured on “stiff” substrata, representing tumor tissue, showed a nearly 14-fold increase in multinucleation compared to cells cultured on “soft”

substrata, representing normal tissue. Multinucleation was regulated in part by signaling downstream of matrix metalloproteinase-3 (MMP3), a protease responsible for remodeling of the extracellular matrix, and commonly found upregulated in cancer. This signaling depended on expression of the Rac1 splice variant, Rac1b, production of reactive oxygen species, and expression of Snail. Under all conditions, cells cultured on soft substrata maintained a low frequency of multinucleation.

Conclusions: Mammary epithelial cells show increased levels of multinucleation with increasing substratum stiffness. This trend suggests that abnormal mechanical characteristics of the tumor microenvironment promote genomic instability in cancer. One responsible mechanism is induced by MMP3; regulation of each component of the pathway shows a direct relationship to multinucleation. This pathway is inhibited on soft substrata, which protects against multinucleation. These data suggest that a drug that promotes the normalization of the microenvironment may be promising.

P1907

Board Number: B418

Understanding the role of protein palmitoylation during asymmetric cell division.

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Generating cellular heterogeneity is fundamental for the development of multicellular organisms and tissue homeostasis. A mechanism of generating cellular diversity is through polarized cell division (or asymmetric cell divisions), where daughter cells acquire distinct identities through the unequal inheritance of cell-fate determinant proteins. Stem cells undergo asymmetric cell division to produce a self-renewing stem cell and a cell fated to differentiate. What remains unclear is how the cell fate determinant asymmetry is established. Extrinsic cues, such as signaling between cells, induce cell polarity during division. Protein asymmetry can also be established through intrinsic cues, where cells become polarized through the segregation of fate determinants before mitosis. The core polarity complex, consisting of CDC42-Par3-aPKC ζ , is asymmetrically localized to the plasma membrane in dividing progenitor cells and regulates the asymmetric partitioning of fate determinants. What remains to be determined is whether the machinery required for membrane localization is essential for establishing polarity. We propose protein palmitoylation, which is a reversible lipid modification that promotes shuttling of proteins between the membrane and the cytosol, is an attractive mechanism of regulating cell polarity and self-renewal. Similar to normal tissue, tumors consist of functionally heterogeneous cell populations differing in proliferative potential, therapeutic resistance, and apoptotic potential. Tumor heterogeneity may be maintained by tumor-initiating cells, a rare population of cells with stem-like potential. Using cancer cell lines to model generation of cellular heterogeneity through asymmetric cell divisions, we find the cell-fate determinant proteins β -catenin and Numb are asymmetrically localized during mitosis. The de-palmitoylating enzyme, APT1 is also asymmetrically localized and inhibition of APT1 disrupts the asymmetric localization of β -catenin and Numb. Our results implicate the core polarity machinery, specifically CDC42, in regulating APT1 asymmetric localization and in turn β -catenin and Numb asymmetry. To understand the role of asymmetric cell division in generating

tumor heterogeneity, we over-express APT1 in breast cancer cells and find these cells have increased self-renewal potential in serial replating assays. Furthermore, we find the self-renewal potential of APT1 over-expressing cells is Wnt-dependent. To characterized changes in cell populations we are examining expression of self-renewal genes and sorting for markers of tumor initiating cells. Taken together, our findings provide a role for palmitoylation during asymmetric cell division and cell-fate decisions in tumors.

P1908

Board Number: B419

FOCAL ADHESION KINASE INHIBITION PREVENTS MELANOMA METASTASIS TO LYMPH NODES.

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Tumor metastasis, as opposed to primary tumor growth, is a major cause of cancer mortality. Interactions between tumor cells and stroma are critical in regulating cancer metastasis. Malignant melanoma would typically metastasize to lymph nodes as a primary or in-transit lesion before secondary metastasis occurs and lymph node biopsy is a common procedure to diagnose melanoma progression. However, the exact mechanism of melanoma metastasis to lymph nodes remains unknown. Recently, we found a new role of focal adhesion kinase (FAK) in regulating inflammatory vascular cell adhesion molecule-1 (VCAM-1) expression, which promotes leukocyte recruitment via $\alpha 4$ integrin interaction. Since malignant melanoma expresses $\alpha 4$ integrin, we hypothesized that lymph nodes express high levels of VCAM-1 due to immune cell trafficking and in turn, it can promote melanoma homing to lymph nodes. First, we tested if FAK activity is required for VCAM-1 expression in lymphatic endothelial cells (ECs) upon tumor necrosis factor- α (TNF- α) treatment to promote melanoma adhesion and transmigration. A pharmacological FAK inhibitor (PF-271, Pfizer; also named as VS-6062, Verastem) blocked TNF- α -mediated VCAM-1 expression on human dermal lymphatic ECs. PF-271 decreased B16BL6 melanoma adhesion and transmigration activity by up to 75% compared to TNF- α treated group. Importantly, oral PF-271 treatment in mice prevented VCAM-1 expression in cervical and popliteal lymph nodes compared to the untreated group. B16BL6 melanoma was injected into the right footpad of C57BL6 mice, and primary tumor growth was allowed for 1 week. Untreated mice exhibited a significant increase in both secondary melanoma growth and in signs of inflammation while PF-271 treated mice (30 mg/kg, twice daily for 1 week) only showed marginal melanoma metastasis. Reduced FAK pY397 autophosphorylation was verified with heart and lung tissues in PF-271 treated group. These results support the possibility that FAK inhibition may be a novel preventative opportunity in melanoma metastasis by blocking inflammatory VCAM-1 expression in lymphatic ECs and lymph nodes.

P1909**Board Number: B420****The role of ITGA1 in pancreatic cancer.**S. Kim¹, Y. Adamian¹, D. Brambilla¹, M. Hoover¹, J. Kelber¹;¹Biology, California State University, Northridge, Northridge, CA

Pancreatic ductal adenocarcinoma (PDAC) is currently the fourth leading cause of cancer-related deaths in the United States and the typical five-year survival rate for patients is less than 5%. PDAC is an invasive malignancy that is difficult to detect early and commonly resistant to therapeutic interventions. Thus, a more complete profiling of molecular regulators in PDAC during disease progression will aid in making novel discoveries to improve diagnostic and treatment methods. Both local invasion and systemic metastases from epithelial-based cancers are driven by pseudopodia/invadopodia formation and function. In this regard, we previously identified PEAK1 (Pseudopodium-Enriched Atypical Kinase One) as a novel regulator of tumor growth/metastasis and therapy resistance in human cancers. To identify co-regulators of pseudopodia formation and pancreatic cancer progression, we cross-referenced our previously published list of pseudopodium-enriched (PDE) proteins with the Oncomine and Babelomics online resources, allowing us to identify novel genes that have yet to be characterized in pancreatic cancer. Of these novel genes, ITGA1 (a cell surface receptor for collagen) is significantly upregulated in pancreatic cancer, yet there are no published reports on the functions of this gene in PDAC. Interestingly, we also found that the expression of ITGA1 increases in PDAC tissues that harbor inactivating mutations in the Smad4 gene, a common genetic alteration in later stages of PDAC. Our molecular analysis of PDAC cells indicates that ITGA1 is expressed at the highest levels in KRas-mutant lines that display a less differentiated morphology. We also found that ITGA1hi PDAC cells are enriched for ALDH1 activity – an indicator of stem-like properties. We evaluated changes in cell cycle profiles of four PDAC cell lines on different ECM substrates in response to various arresting agents. Interestingly, PANC1 cells underwent a significant induction of apoptosis when grown under serum starved conditions on fibronectin or laminin substrates. However, collagen inhibited this serum-starvation induced apoptotic response suggesting that ITGA1 may promote the survival of KRas-mutant PDAC cells when exposed to extracellular stress (e.g., nutrient deprivation). Thus, targeting cell-surface ITGA1 may be a viable mechanism for inhibiting PDAC cell proliferation. Future studies will evaluate the function of ITGA1 in PDAC cells using RNAi and/or immunoneutralizing methods.

P1910**Board Number: B421****Fibronectin enriched environment promotes single cell migration of high invasive Oral Squamous Cell Carcinoma.**M.L. Lamers^{1,2}, L. Bernardi³, G.d. Ramos³, M. Sant'Ana Filho³, A.R. Horwitz⁴;¹University of Virginia, Charlottesville, VA, ²Dept of Morphological Sciences, Federal University of Rio Grande do Sul, Porto Alegre, Brazil, ³Oral Pathology, Federal University of Rio Grande do Sul, Porto

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Oral Squamous cell carcinoma (OSCC) is an epithelial neoplasm that has a low patient survival rate mainly due to the highly invasive and metastatic characteristics of this tumor. Tissue invasion and metastasis depends on the adaptation of the tumor migratory abilities to new environments, e.g., the switch between the laminin (LN) enriched composition of the epithelia basal membrane to collagen and fibronectin (FN) enriched connective tissue. The aim of this study was to analyze the role of this compositional change on the migratory behavior of cells from an oral squamous cell carcinoma. OSCC cell lines - Cal27 (high E-cadherin) and SCC25 (low E-cadherin) - were plated in 2D or 3D environment with different ECM composition, and the overall migratory properties were accessed using time-lapse microscopy. In 2D conditions, both lines migrated in collectives when plated on either Matrigel (50ml/cm²) or LN (2ug/ml); while FN (2ug/ml) produced a more rapid, single cell migration mode only in the low differentiated cell line (SCC25), a behavior that was also observed in a more complex 3D matrix (collagen 1.2mg/ml + FN 2ug/ml). To analyze the effects of ECM composition on signals that regulate cell migration, the levels of activated Rac1 were measured in cells plated on LN or FN (2ug/ml). The SCC25 cells showed intrinsically higher Rac1 activated levels when compared to the highly differentiated OSCC (Cal27) cells, and the composition of ECM did not substantially affect the activation levels. Since the observed ECM-related changes in cell migration might be due to a difference in adhesion, we analyzed their morphology and turnover using paxillin, vinculin and focal adhesion kinase as markers. Both cell lines showed highly elongated adhesions when plated on LN; however, the high invasive OSCC showed a switch to smaller and more dynamic adhesions when plated on FN. In order to translate these *in vitro* results regarding ECM-mediated tumor adhesion changes, immunostaining of human OSCC biopsies showed that leading low E-cadherin invasive cells in the FN enriched connective tissue had a more frequent distribution of these integrin-related adhesion molecules at the cell border when compared to the high E-cadherin epithelia adjacent to the tumor. We show that the differentiation level of the OSCC tumor determines the response to the ECM composition and influence the tumor migratory behavior by altering the mode from collective to single cell migration, likely due to changes in cell-ECM adhesion properties induced by fibronectin.

P1911

Board Number: B422

Role of Myosin II isoforms in MDA-MB-231 cell mediated matrix rearrangement.

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The extracellular matrix (ECM) has been shown to play a role in normal tissue development, as well as in the pathogenesis of certain diseases. Changes in ECM stiffness result in changes in cellular tension and stiffness, which in turn result in changes in cell signaling and proliferation. This delicate balance is known as mechanoreciprocity, and loss of this tensional homeostasis can promote the growth of many solid

tumors. In the case of breast cancer, it has been shown that a stiffer matrix promotes cancer progression. Nonmuscle myosin II, the main force generating protein in nonmuscle cells, may be important for the cell mediated matrix changes that occur during tumorigenesis. To determine the importance of Myosin II isoforms in regulating matrix alteration by cancer cells, we depleted MDA-MB-231 cells, an aggressive and metastatic breast cancer cell line, of specific Myosin II isoforms. shRNAs targeting nonmuscle myosin IIA and IIB heavy chains were used to make stable IIA knockdown (IIA KD) and IIB knockdown (IIB KD) MDA-MB-231 cell lines. IIA and IIB protein levels were reduced by 80-95% of vector controls and protein levels were routinely monitored to ensure myosin II KD was consistent between experiments. Loss of myosin IIA and IIB had minimal effect on cell morphology in 2-D cultures, while in 3-D cultures morphology was altered compared to parental MDA-MB-231 cells, which exhibited a pyramidal cell body with numerous cell. IIB KD cells were elongated and appeared localized to one focal plane while IIA KD cells were highly branched, sending processes in all directions. To determine if myosin II is needed for MDA-MB-231 cell mediated matrix organization, we measured the ability of cells to compress a collagen matrix at 1 and 4 days post casting. MDA-MB-231 parental cells effectively compressed constructs (to 400 μm from 1 mm) while loss of myosin IIA resulted in an 80% inhibition of gel compression. KD of myosin IIB had minimal effect on gel compression. In addition to gel compression assays, we developed a method to directly measure matrix stiffness using isometric force measurements. Engineered collagen constructs are subjected to a conditioning protocol that is used to calculate the elastic modulus of the constructs. The elastic modulus of parental MDA-MB-231 collagen constructs was 10.3 mPa, while the elastic modulus of IIA was 3.3 mPa, a 3-fold difference from parental constructs. IIB KD constructs were more similar to parental constructs with a modulus of 7.7 mPa. Collagen constructs containing no cells had an elastic modulus of 0.4 mPa. These results indicate myosin II isoform are required for matrix compression and differentially regulated the rigidity of the ECM.

P1912

Board Number: B423

Dynamically Stiffening Hydrogels Promote Malignant Transformation of Mammary Epithelial Cells.

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Mammary epithelial cells, e.g. MCF10A, are known to respond to differences in static ECM stiffness and resemble a mesenchymal, more malignant state on stiffer ECM lacking any polarized acinar structure. While this is akin to the stiff mammary tumors that one can detect with manual palpation, breast cancer fibrosis is a dynamic process that results in matrix stiffness increasing from normal ~ 150 Pa to 1500 Pa over months to years as a result of enhanced collagen expression and lysyloxidase crosslinking. To more accurately mimic the onset of tumor-associated fibrosis, MCF10A mammary epithelial cells were cultured on dynamic methacrylated-hyaluronic acid (MeHA) hydrogels, whose stiffness that can be modulated from “normal” 100 Pa to “malignant” 2000 Pa, utilizing a two step polymerization process.

MCF10A cells cultured on 100 Pa MeHA hydrogels form and remain as polarized acini and then begin to decompose and resemble a mesenchymal morphology upon matrix stiffening to 2000 Pa. However, the degree of matrix stiffening and culture time prior to stiffening play a large role in acini transformation; increasing culture time on soft matrix prior to stiffening from 6 to 10 days delayed the onset malignant transformation by as much as 35%. To ensure that early stiffening was not cell density dependent, preformed acini of differing cell density were transplanted onto stiffening MeHA hydrogels. Decomposition of the acini upon matrix stiffening is independent of acinus size and cell density, suggesting that an individual acinus responds to the stiffness of the underlying substrate but that their epigenetic state and structure can protect a subset of acini from stiffening. This data indicates a more complex interplay of intrinsic ECM cues and acinar structure in regulating signaling that results in epithelial-to-mesenchymal transition and the onset of a malignant phenotype.

P1913

Board Number: B424

Defining how the mechanical and molecular properties of the microenvironment control autophagy in breast cancer cells.

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In order to survive and progress, many tumors “co-opt” macroautophagy (hereafter autophagy), a process through which obsolete, damaged, or otherwise deleterious cytosolic components are transported to and degraded by the lysosomal pathway. Since autophagy and features of the tumor microenvironment are applicable to several types of cancer, here we explored how the molecular and mechanical microenvironment controls autophagy. The microenvironments of solid tumors tend to be fibrotic and have elevated levels of interstitial fluid pressure, both of which promote tumor progression, and both of which are implicated in the induction of autophagy. We investigated the relationship between these mechanical stressors and autophagy by using polyacrylamide gels of different stiffness and in microfluidic chambers that simulated of interstitial flow. The level autophagy in MDA-MB-231 human breast cancer cells was quantified by determining the relative expression and localization of autophagic marker LC3II using immunofluorescence. Conditions that had the strongest effect on autophagy were selected for molecular study, focusing on the phosphoinositol-3 kinase and canonical and non-canonical YAP/TAZ signaling pathways, all of which receive input from biomechanical stimuli and have been shown to play a role in the regulation of autophagy. Our data suggest that the mechanical properties of the tumor microenvironment regulate signaling that controls autophagy.

P1914**Board Number: B425****A Trio of Indices Identifies and Quantifies Heterogeneity in Cellular Systems: Application of Heterogeneity Indices to Drug Discovery and Diagnostics.**A.H. Gough^{1,2}, T.Y. Shun², M.E. Schurdak^{1,2,3}, D.L. Taylor^{1,2,3};¹Computational and Systems Biology, Univ of Pittsburgh, Pittsburgh, PA, ²Drug Discovery Institute, Univ of Pittsburgh, Pittsburgh, PA, ³Cancer Institute, Univ of Pittsburgh, Pittsburgh, PA

One of the greatest challenges in biomedical research, drug discovery and diagnostics is to understand how seemingly identical cells can respond differently to perturbagens, such as drugs for disease treatment. Although heterogeneity is an accepted and common characteristic of a population of cells, it is rarely evaluated or reported. The standard practice for cell-based high content assays, as well as other cell analysis applications, has been to assume a normal distribution and to report an average value with a standard deviation for a cell population which may not be normal distributed. To address this important gap we sought to define a method that could readily be used to identify, quantify and characterize population heterogeneity in assays of both cells and small organisms, and furthermore, to guide decisions in drug discovery, experimental cell/tissue profiling and development of diagnostics. Our study revealed that heterogeneity can be effectively identified and quantified with three indices that indicate diversity, non-normality and fraction of outliers. The indices were evaluated using the induction of STAT3 activation by interleukin-6 (IL-6) and oncostatin M (OSM) and the inhibition of STAT3 activation by known pathway inhibitors in five cell lines, where the systems response, including sample preparation and instrument performance, were well characterized and controlled. Results indicate that the heterogeneity in the activation of STAT3 varies by cell line and pathway of activation. Cal33 cells stimulated with IL-6 exhibit a distinctly bimodal distribution while OSM induces a more normal distribution of STAT3 activity. These responses are clearly identified by an increase in the non-normality index only in the IL-6 treated cells, indicating a multimodal population distribution. In contrast, 686LN and MCF10A cells show nearly identical profiles with apparently low heterogeneity when activated by IL-6 and OSM, and in both cases exhibiting a low non-normality index, statistically indicating a single normally distributed population. Neither MCF-7 nor MB468 show significant activation of STAT3 by IL-6, though in both cases there is a large percent of outlier cells that exhibit a positive IL-6 response, while the majority of both cell types respond to OSM. We will present further applications of these indices to the analysis of heterogeneity in cancer tissue and other cellular systems. Understanding heterogeneity in the response to perturbagens, as well as in cancer tissues, will become a critical factor in designing strategies for the development of therapeutics including targeted polypharmacology.

P1915**Board Number: B426****Transient external force induces phenotypic reversion of malignant epithelial structures via nitric oxide Signaling.**B.L. Ricca¹, G. Venugopalan¹, K. Tanner^{2,3}, S. Furata², W. Orellana^{2,3}, C. Reber¹, D.G. Brownfield^{1,2,4}, M.J. Bissell², D.A. Fletcher^{1,5,6};¹Department of Bioengineering, University of California, Berkeley, Berkeley, CA, ²Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, ³Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, ⁴Biochemistry, Stanford University, Stanford, CA, ⁵Biophysics Graduate Group, University of California, Berkeley, Berkeley, CA, ⁶Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA

Single breast epithelial cells embedded in three-dimensional laminin-rich extracellular matrix (ECM) gels grow to form highly organized, growth-arrested structures known as acini. In contrast, malignant cells form larger, disorganized structures that grow continuously and have no lumen or apical-basal polarity. The malignant cells can be phenotypically 'reverted' by treatment with inhibitory compounds and antibodies at the single cell stage, leading them to form organized, growth-arrested structures that resemble the non-malignant acini. Here, we investigate the effects of external compression on acinar morphogenesis from both malignant and non-malignant cells and find that phenotypic 'reversion' can be accomplished mechanically. Using an elastic chamber to apply a compressive strain that generates a transient, quickly relaxing force, we show that compressed single malignant cells form growth-arrested, polarized, acinar-like structures by day 10. This phenotypic 'reversion' of malignant cells under compression is distinct in that it does not require use of exogenous molecular inhibitors/activators. We find that compressive strain stimulates nitric oxide production in breast epithelial cells and that inhibiting nitric oxide production blocks sensitivity to compression, indicating that mechanically-induced reversion proceeds via nitric oxide signaling pathways. Time-lapse microscopy of the malignant cells after compression revealed that compression restored coherent rotation of the cells, a behavior critical for acinar development but lost in malignancy. We propose that external forces modulate the acinar morphogenesis program by altering nitric oxide signaling early in development, with profound consequences for final tissue architecture.

P1916**Board Number: B427****Detection and Characterization of Infiltrating Immune Cells in Breast Cancer Tissue using Multicolor Immunohistochemistry.**S.G. Becker-Catania¹, X. Li¹, L. Ma¹, B. Atteberry¹, Q. Huang¹, P. Just¹;¹Research and Development, eBioscience, An Affymetrix Company, San Diego, CA

Breast cancer is a highly heterogeneous disease divided into four major subtypes, the identification of which is important for treatment strategy and clinical outcome. Cancers are classified into specific subtypes using hormone receptor status, levels of HER2/neu expression and proliferation rate. In addition to subtype, recent publications have documented the presence of infiltrating leukocytes within the tumor and the importance of these cells in modulating the host immune response in the tumor microenvironment. Identification and characterization of these populations of infiltrating immune cells has been limited by the number of proteins that can be examined within a single tissue section using standard colorimetric immunohistochemistry. To address this limitation, we have used panels of fluorophore-conjugated antibodies to both classic breast cancer markers such as PR, HER2, cytokeratin, mucin 1, and Ki-67, as well as immune cell antigens to stain formalin-fixed paraffin embedded tissue sections from infiltrating ductal carcinoma cases. We found cytotoxic T cells (CD8+), T helper cells (CD4+), regulatory T cells (CD4+/Foxp3+), macrophage (Ham56+), and CD45RB+ cells migrating within the tumor stroma of luminal breast cancer cases. The presence of both cytotoxic T cells and T helper cells that are known to activate anti-tumor immunity as well as regulatory T cells thought to suppress anti-tumor immunity illustrates the complexity of the tumor microenvironment and the need to multiplex markers in order to better characterize and understand the immune response in individual patients.

P1917**Board Number: B428****Pancreatic Cancer Progression Mediated by Alternatively Spliced Tissue Factor under Normoxic and Hypoxic Conditions.**D. Ramchandani¹, D. Unruh², V.Y. Bogdanov², G.F. Weber¹;¹James L. Winkle College of Pharmacy, University of Cincinnati, Cincinnati, OH, ²College of Medicine, University of Cincinnati, Cincinnati, OH

Background: Tumor progression is mediated by aberrant expression or splicing of a unique set of genes. Tissue factor is an integral membrane protein that serves as the initiator of blood coagulation. An alternatively spliced form of tissue factor (asTF) is generated by deletion of exon 5 and a resultant frameshift in exon 6, yielding a secreted protein. High expression of asTF in pancreatic tumor cells was previously reported; recently, asTF was found in abundance in pancreatic ductal adenocarcinoma lesions and lymph node metastases. asTF binds $\alpha 1$ integrins in a non-canonical region, acting as an integrin

ligand that drives pro-survival signaling pathways and metastatic spread in vivo. However, it remains unknown whether hypoxic conditions affect asTF's ability to fuel pancreatic tumor progression. **Methods:** The high grade pancreatic ductal adenocarcinoma cell line PT45P1 and its previously described asTF-overexpressing subline PT45/asTF+ were analyzed for proliferation, anti-anoikis, and motility under normoxic conditions (5% CO₂, ambient O₂, 1000 mg/ml glucose) that model early stages of cancer, and hypoxic conditions (20% CO₂, 1% O₂ and 5 mM lactate) – a characteristic of advanced malignant lesions. **Results:** asTF-overexpressing PT45P1/asTF+ cells displayed higher proliferation rates compared to PT45P1 cells. PT45/asTF+ cells retained their higher growth ability in both normoxic as well as hypoxic environments. By propidium iodide staining, the percentage of PT45/asTF+ cells in the G₂/M phase of the cell cycle was significantly elevated, whereas the majority of the PT45P1 cells remained in the quiescent G₀/G₁ phase. Similarly, under de-adherent conditions (plating on poly-HEMA) that mimic tumor spread through the vasculature, the percentage of PT45P1/asTF+ cells in G₂/M phase was significantly higher than that of PT45P1 cells under normoxic and hypoxic conditions. Interestingly, PT45P1/asTF+ cells expressed higher levels of carbonic anhydrase 9 (CAIX), a hypoxia-inducible enzyme that plays key roles in maintaining intracellular pH and increasing cancer cell invasiveness. Finally, asTF-overexpression also imparted increased directional motility in a gap closure assay. **Conclusion:** The elevated expression of asTF in pancreatic cancer enhances tumor growth and spread. The effects are sustained in the normoxic conditions of an early cancer and the hypoxic conditions that exist in late stages, likely at least in part through the induction of CAIX. Molecularly targeted treatment strategies have thus far focused on suppressing mutated oncogenes. Alternatively spliced gene products of tumor progression may also be valid drug targets.

P1918

Board Number: B429

Phenotyping and functional studies of bone marrow stromal cells in hematological malignant diseases.

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Background: It is now widely recognized that bone marrow stromal cells (BMMSCs) play an important role in the pathogenesis of several hematological malignancies. BMMSCs have low immunogenicity and are capable of inhibiting the proliferation and activation of T lymphocytes. These effects are mediated by cell-cell contact and soluble factors. Nevertheless, the phenotypical characteristics and biological functions of BMMSCs from patients with myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML) remain unclear. **Aims:** To characterize the phenotype, immunosuppressive ability and expression of *TGFβ1*, *IDO*, *RPGE2*, *IL1β* and *IL6* in BMMSCs from MDS and AML patients. **Methods:** At the onset of

the disease and after informed consent, bone marrow (BM) samples were collected from 17 MDS patients (aged from 50 to 83) and 7 AML patients (aged from 30 to 86). Seven healthy donors (aged from 28 to 57) were used as controls. BMMSCs were analyzed always after the fourth passage. Flow cytometry immunophenotyping was performed using the following antibodies: CD31, CD34, CD45, HLA-DR, CD73, CD90 and CD105. For the T cell proliferation assay, allogeneic CFSE-labeled CD3⁺ T lymphocytes were cultured at BMMSC/Tcells ratio of 1/2, 1/5, 1/10, 1/50 and 1/100. Stimulation of T lymphocytes was induced by adding PHA. Four days later CFSE fluorescence intensity was analyzed by flow cytometry. The relative gene expression was determined by quantitative PCR. Mann Whitney and ANOVA were used for statistical analysis. **Results:** Although MDS and AML derived-BMMSCs were similar in phenotype to the healthy donor group, the immunosuppressive effects of AML-BMMSCs on CD3⁺ T-cell proliferation were abolished. AML-derived BMMSCs presented a significant increase in expression levels of *IDO*, *IL1β* and *IL6* when compared with the healthy group. In contrast, MDS derived-BMMSCs were similar to the control group in their ability to inhibit the proliferative response of CD3⁺ T lymphocytes ($P < 0.01$), and there were no significant differences in any of the cytokines tested between these groups. **Conclusions:** The production of IDO, IL6 and IL1β by AML blasts has been demonstrated, however we have shown for the first time those cytokines being produced by AML derived-BMMSCs. IDO is an immunosuppressive agent that blocks T-cell activation in AML. The presence of pro-inflammatory cytokines IL6 and IL1β comprises a hallmark of cancer-related inflammation. Our findings indicate that although AML derived-BMMSCs are similar in phenotype to the healthy group, they lose their ability of inhibiting T-lymphocytes proliferation, possibly contributing to tumor growth.

P1919

Board Number: B430

Inflammatory Cytokines Promote Melanoma Proliferation and Migration.

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Experimental and clinical data indicate that obesity increases the risk for colorectal, esophageal, pancreatic, endometrial, renal cell carcinoma, and post-menopausal breast cancer. We found that melanoma grows much faster in obese mice than in normal body weight mice. Several metabolic, endocrine, and immune system factors are positively correlated with a poor prognosis and shorter disease-free survival times in obesity associated cancers. The paracrine signaling between tumor cells and stromal cells is a hallmark of cancer that has only recently become appreciated as a catalyst for mutagenesis, immune evasion, and metastasis. Although some mechanistic knowledge has been obtained from a “one growth factor, one effect” approach, this must be paired with a systems analysis to gain an understanding of the interactions between stromal cells and melanoma cells. Our data reveals that the inflammatory cytokines, resistin and IL-6, promote melanoma proliferation (6.1×10^5 cells/day and 1.0×10^6 cells/day v. 5.5×10^5 cells/day for controls, $p = 0.05$). There is still a wide gap in our knowledge of how obesity promotes melanoma metastasis. Individual inflammatory cytokines had no effect on melanoma migration, but co-culturing cells with macrophages significantly increases migration.

Preliminary data also suggests that co-culture with adipocytes increases melanoma migration. The influence of inflammation on tumors is well known, but the mechanisms by which fat cells affect melanomas is unknown. These results suggest that there could be many stromal cells that affect melanoma growth and metastasis in obese individuals. This work was supported by the Mississippi INBRE, funded by an Institutional Development Award (IDeA) from the National Institute of General Medical Sciences of the National Institutes of Health under grant number P20GM103476.

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P1920

Board Number: B431

HERC2/USP20 coordinates CHK1 activation by modulating CLASPIN stability.

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CLASPIN is an essential mediator in the DNA replication checkpoint, responsible for ATR (ataxia telangiectasia and Rad3-related protein)-dependent activation of CHK1 (checkpoint kinase 1). Here we found a dynamic signaling pathway that regulates CLASPIN turn over. Under unperturbed conditions, the E3 ubiquitin ligase HERC2 regulates the stability of the deubiquitinating enzyme USP20 by promoting ubiquitination-mediated proteasomal degradation. Under replication stress, ATR-mediated phosphorylation of USP20 results in the disassociation of HERC2 from USP20. USP20 in turn deubiquitinates K48-linked-polyubiquitinated CLASPIN, stabilizing CLASPIN and ultimately promoting CHK1 phosphorylation and CHK1-directed checkpoint activation. Inhibition of USP20 expression promotes chromosome instability and xenograft tumor growth. Taken together, our findings demonstrated a novel function of HERC2/USP20 in coordinating CHK1 activation by modulating CLASPIN stability, which ultimately promotes genome stability and suppresses tumor growth.

P1921

Board Number: B432

Predicting the Effects of Clinically Observed Kinase Mutations using Molecular Modeling and Machine Learning Algorithms.

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Many cellular processes are impacted by signaling through receptor and non-receptor kinase proteins. These include such diverse cellular actions as proliferation, differentiation, and motility, as well as tissue level phenomena such as angiogenesis and development. This important role in the cell is reflected also

in the relative overrepresentation of kinases among known cancer mutations to proteins. Through database mining, we have found that kinase domain mutations represent over 20% of somatic mutations in one cancer mutation database, COSMIC. In order to better understand the functional effects of these mutations, we have developed computational methods that seek to predict the effect of point mutations on kinase activation. By predicting whether a given mutation causes a kinase to be more active, we can gain insight into the overall impact of the mutation on cell phenotype as well as give insight to clinicians on patient cohorting for efficacious treatment with targeted kinase inhibitors. We have developed two separate but complementary methods to predict kinase activation status. The first uses molecular dynamics (MD) simulations and scoring criteria to predict if a mutation preferentially stabilizes the protein's active state. This has proven accurate for predicting the effects of intracellular kinase domain mutations of anaplastic lymphoma kinase (ALK), and has also been extended to the epidermal growth factor receptor (EGFR). We believe that the success of this method on kinases with diverse activation methods (EGFR signals through allosteric activation via homo- or heterodimeric complexes, while ALK is thought to signal through activation by trans-autophosphorylation of activation loop tyrosine) shows that this is a generally applicable method. As a complimentary approach to MD, we have developed machine learning techniques that utilize the method known as support vector machines to predict whether mutations in a large number of kinases (>300) are activating. This method has shown great promise as it can correctly distinguish between an activating mutation and one that is not activating almost 90% of the time on cross-validation. Also, this method has proven to be almost as effective at predicting activation mutations as the mechanistic picture gained from MD simulations. We think these methods are both broadly applicable and have the potential to greatly impact both our understanding of mechanisms of kinase activation as well as to guide best practices in the clinical setting of targeted therapy in cancer treatment.

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P1922

Board Number: B433

Persistent STAT3 signaling contributes to the resistance of anti-cancer drugs doxorubicin and cisplatin, and MEK inhibitor AZD6244 in human sarcoma cells.

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Signal Transducer and Activator of Transcription 3 (STAT3) signaling pathway is activated in response to cytokines and growth factors. Persistently activated STAT3 are frequently detected in many types of human cancers and are believed to play an important role in their oncogenesis. Stat3 has been classified as a proto-oncogene because activated STAT3 can mediate oncogenic transformation in cultured cells and tumor formation in nude mice. We previously demonstrated that persistently activated STAT3 is frequently detected in human sarcoma such as rhabdomyosarcoma. To further

examine the role of persistent STAT3 signaling plays in rhabdomyosarcoma, we used genetic approaches to either knock-down the expression of STAT3 using short hairpin RNA (ShRNA) or expressed constitutive active STAT3 protein. Knock-down the expression of STAT3 in sarcoma cells that express persistent STAT3 signaling, reduced the cell viability and sensitized cells to anti-cancer drugs doxorubicin and cisplatin. On the other hand, expression of constitutive active STAT3 protein in sarcoma cell lines lacking persistent STAT3 signaling decreased the sensitivity of those cells to anti-cancer drugs doxorubicin and cisplatin. Furthermore, a MEK inhibitor, AZD6244 that is being tested in human cancer clinical trials unexpectedly induced STAT3 phosphorylation suggesting inducing of STAT3 may induce intrinsic resistance to MEK inhibitor. Accordingly, knock down of STAT3 sensitize sarcoma cells to MEK inhibitor treatments.

To further translate our finding to potential cancer therapy, we tested a small molecular STAT3-selective inhibitor LY5 in sarcoma cells expressing persistent STAT3 signaling. Our data demonstrated that LY5 inhibited STAT3 phosphorylation, STAT3 DNA binding activity, and the migration of sarcoma cells. In addition, the combination of LY5 with anti-cancer drugs doxorubicin and cisplatin and the MEK inhibitor, AZD6244 also shown stronger inhibitory effects than single agent alone. In summary, our results using both genetic and small molecule approaches demonstrated that persistent STAT3 signaling contributes to the resistance of anti-cancer drugs doxorubicin and cisplatin, and MEK inhibitor in human sarcoma cells. They also implicated a potential cancer therapy strategy with the combination of STAT3 inhibitor with doxorubicin, cisplatin, and MEK inhibitor in sarcoma cells expressing persistent STAT3 signaling.

P1923

Board Number: B434

LONG NON-CODING RNAs IN BREAST CANCER PREVENTION.

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Our initial transcriptomic analysis of normal breast of parous and nulliparous postmenopausal women reveals that long non-coding RNA genes (lncRNAs) are up-regulated in the parous breast [Int. J. Cancer: 131, 1059-1070, 2012]. This observation provided a new paradigm in the understanding of the role of lncRNAs in the regulation of transcription and their potential function in pregnancy's preventive effect in reducing the lifetime risk of developing breast cancer. lncRNAs are RNA molecules longer than 200 nucleotides that are not translated into proteins, but regulate the transcription of genes involved in different cellular processes, including differentiation, cancer initiation and progression. The relevance of lncRNAs in the transcription field is beginning to be explored and their roles have been found to vary from guiding proteins to the genome to scaffolding proteins complexes needed for the transcription of a specific gene. In this work, RNA sequencing of healthy postmenopausal breast tissue biopsies from 8 parous and 8 nulliparous women using Illumina platform was performed. The sequencing results show that there are 42 lncRNAs differentially expressed between parous and nulliparous breast tissue. After analysis of these 42 lncRNAs using bioinformatic tools, scientific literature, and thermodynamic filters, thirteen lncRNAs have been selected to be tested in vitro. Using RT-qPCR we have determined that

eleven out of these thirteen lncRNAs are expressed in a variety of epithelial breast cancer cell lines (luminal, basal and HER2+ cells). Seven lncRNAs are upregulated and two are downregulated in breast cancer cell lines compared to normal-like cells. Also, two of the lncRNAs are not expressed in any of the three normal-like cells tested and three lncRNAs are only expressed in either luminal or basal cell lines. These results indicate that these lncRNAs may be key targets to study and potentially treat different types of breast cancers. This work provides not only novel information on lncRNAs induced by pregnancy in breast cells, but also places lncRNAs as potential key regulators in breast differentiation, cancer initiation and cancer progression. Determining their functions will render a comprehensive picture of their role in the human breast at the transcriptomic level, and will lead to the identification of key lncRNAs driving breast transformation. Furthermore, the manipulation of these lncRNAs can eventually lead to the development of therapeutics for breast cancer prevention. (This work was supported by NIH core grant CA06927 to Fox Chase Cancer Center)

P1924

Board Number: B435

Epstein-Barr Viral and Human microRNAs Target Cell Cycle and Immunoregulatory Human Transcripts.

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Epstein-Barr virus (EBV) is a human herpesvirus that chronically infects ~95% of the worldwide population. EBV infection can lead to the development of specific cancers including nasopharyngeal carcinoma (NPC) and Burkitt's lymphoma (BL), the latter of which is the focus of this research. MicroRNAs (miRNAs) are ~22 nt, non-coding RNA molecules that imperfectly base pair to mRNAs to repress translation. EBV produces 49 mature miRNAs that can accumulate to ~25% of total active miRNAs in infected BL cells. Our research aims to match viral miRNAs with human transcripts to better understand the mechanism of the disease and identify potential therapeutic targets. Previous high-throughput biochemical/bioinformatic experiments using HITS-CLIP identified likely human mRNA targets in the 3' UTRs of genes with a variety of important cellular functions in the context of BL cells. Using luciferase reporter assays, we demonstrate that three human transcripts involved in cell cycle progression and immunoregulation are regulated by EBV miRNAs, human miRNAs, or both. Together these results provide preliminary evidence for the involvement of EBV miRNA regulation in diverse cellular pathways.

P1925

Board Number: B436

Comparative analysis of the PDZ domain-containing targets of the E6 oncoproteins from diverse high-risk human papillomavirus types.

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Human papillomavirus (HPV) types that are defined, to varying degrees, as cancer-causing, have a C-terminal, 4-amino acid PDZ Binding Motif (PBM) on the E6 oncoprotein; those types that are not associated with cancer have no PBM. More than ten cellular PDZ-containing proteins have been defined as bona fide targets of E6, but not all are recognised by all oncogenic E6s. Furthermore, structural and mutational analyses of PDZ/PBM interactions have shown that the amino acid residues upstream of the PBM, which are quite diverse in different HPV types, are involved in PDZ target selection. These data suggest that E6s from different oncogenic HPV types might have individual fingerprints, with respect to their PDZ-containing targets. Using biotinylated peptides corresponding to the C-terminal 10 amino acids of the E6 proteins from a number of HPV types, we have performed pulldowns from epithelial cells and subjected these to mass spectroscopic analysis. The results confirm hDlg as the major PDZ-containing target of all cancer-causing HPV E6 proteins, but certain differences in the selection of other PDZ-containing targets appear to correlate with the degree of cancer-association reported for the various HPV types. We are now validating and extending these findings in biochemical and cellular assays.

P1926

Board Number: B437

Whole genome expression analysis of Cox4i1 knockout cells indicates increased glycolysis and carcinogenesis.

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Cytochrome c oxidase (CcO) complex is an important enzyme for ATP synthesis in the electron transport chain of mitochondria. Here we studied the effect of CcO pathway disruption on glycolysis and carcinogenesis using whole genome microarray expression analysis of a mouse cell line with cytochrome c oxidase subunit 4 isoform 1 (Cox4i1) silenced by short hairpin RNA. The expression of Cox4i1 in the knockout was less than 5 percent of the control (p-value less than 10 to -4), indicating the successful disruption of the CcO pathway. 130 genes changed significantly between the knockout and the control. We did function and pathway analysis on those genes. Among those genes, we found that more than 50 glycolysis related genes were up regulated by more than 4-fold with an enrichment p-value less than 10 to -6. We also found that more than 20 genes related to pathways in cancer were up regulated by more than 4-fold with an enrichment p-value less than 10 to -4. These results indicate that the disruption of CcO pathway increases the glycolysis-dependent ATP synthesis and that there might be a causal effect of glycolysis on carcinogenesis.

P1927**Board Number: B438****SWAP-70 contributes to spontaneous transformation of mouse embryo fibroblasts.**Y. Fukui¹, K. Morishita², T. Ichikawa², R. Jessberger³;¹Institute of cellular and system medison, National Hlth Res Inst, Zhunan Town, Taiwan, ²Faculty of Medicine, University of Miyazaki, Miyazaki, Japan, ³Medical Theoretical Center, Technical University of Dresden, Dresden, Germany

Mouse embryo fibroblasts (MEFs) grow slowly after cultivation from animals, however, after an extended period of cultivation, their growth accelerates. We found that SWAP-70 deficient MEFs failed to increase growth rates. They maintain normal growth rates and proliferation cycles for at least 5 years. Complementing SWAP-70 deficiency in one of these MEFs, MEF1F2, by expressing human SWAP-70 resulted in fast growth of the cells after further cultivation for a long period. The resulting cells show a transformation phenotype, since they grow on top of each other and do not show contact inhibition. This phenotype was reverted when sangunarine, a SWAP-70 inhibitor, was added. Two SWAP-70 expressing clones were examined in detail. Even after cell density became very high their cdc2 and NFkB were still activated suggesting that they do not stop growing. One of the clone formed colonies in soft agar and formed tumors in nude mice, but the other did not. Lately, one more clone became transformed being able to make colonies in soft agar. We maintain 4 human SWAP-70 expressing MEF1F2 cell lines. Three out of 4 clones exhibited transforming phenotypes. The mouse SWAP-70 gene also promoted transformation of MEFs. Taken together our data suggest that SWAP-70 is not a typical oncogene, but is required for spontaneous transformation of MEFs.

P1928**Board Number: B439****Anti-neoplastic drugs at sub lethal doses augment caveolin-1 expression and caveolin-1 dependent migration and invasión of cancer cells.**N. Diaz^{1,2,3}, C. Calderon¹, J. Díaz^{1,2,4}, H. Maldonado^{1,3}, J. Castillo^{1,2}, R. Ortiz^{1,2,3}, L. Leyton^{1,2,3}, A. Quest^{1,2};¹Laboratory of Cellular Communication, Center for Molecular Studies of the Cell (CEMC), University of Chile, Santiago, Chile, ²Advanced Center for Chronic Diseases (ACCDiS), University of Chile, Santiago, Chile, ³Biomedical Neuroscience Institute (BNI), Cell and Molecular Biology Program, Biomedical Sciences Institute (ICBM), University of Chile, Santiago, Chile, ⁴Laboratory of Cellular and Molecular Biology, Department of Basic and communitarian Sciences, University of Chile, Santiago, Chile

Introduction: The key to successful cancer therapy resides in identifying the elements required within a functional oncogenic network that sustain tumor cell viability. Selective inhibition of those elements should then lead to specific elimination of tumor cells. Although some advances have been made in designing more effective treatments, relatively non-specific chemotherapeutic approaches remain the

major therapeutic tool for treating tumors despite the existence of adverse secondary effects and the development of multi-drug resistance, which both significantly reduce the efficacy of such approaches. Here we evaluated whether these treatments may alter the expression of caveolin-1 (CAV1), and favor metastasis. CAV1 plays a dual role in tumor progression and although considered a tumor suppressor at early stages of tumor development, elevated CAV1 levels are associated with metastasis and multidrug resistance in later stages of cancer. Specifically the phosphorylation of CAV1 on tyrosine 14 (Y14) by Src family kinases has been shown to increase cell migration and invasion of tumor cells. However the mechanisms that may lead to upregulation of CAV1 levels in cancer cells and whether such events may be triggered by chemotherapeutic drugs remain unclear. Here we investigated whether the treatment of cancer cells expressing low endogenous CAV1 levels with different chemotherapeutic agents induced CAV1 up-regulation and phosphorylation on Y14, as well as increased migration and invasion of colon cancer cells. Furthermore, we evaluated the signaling pathways involved in drug-induced CAV1 upregulation. Methodology: The colon cancer cell lines DLD1 and HT29(US) were treated with the anti-neoplastic drugs Methotrexate (MTX) or Etoposide (ET) and expression of CAV1 was analyzed by western blotting. To identify pathways involved in the induction of CAV1, cells were pre-treated with the MEK inhibitor (PD98059), the Src family inhibitor (PP2) or the anti-oxidant Trolox prior to MTX or ET exposure. Phosphorylation of MAPK and ROS levels were determined by western blotting and flow cytometry, respectively. Additionally, cell migration and invasion were evaluated in transwell and matrigel assays. Results: In DLD1 and HT29(US) cells, CAV1 expression, migration and invasiveness increased upon treatment with anti-neoplastic drugs. CAV1 upregulation was mediated by a sequence of events including activation of the MEK/ERK pathway and ROS production. Drug-enhanced migration and invasion of the cancer cells was linked to CAV1 phosphorylation on tyrosine-14 therefore MTX and ET increased CAV1 expression by a pathway involving MEK/ERK activation and ROS production. Increased phosphorylation on tyrosine-14 lead to increased migration and invasiveness of colon cancer cells.

P1929

Board Number: B440

The Effects of Estradiol and Bisphenol A (BPA) on Histone Modifying Enzymes in Ovarian Cancer.

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Ovarian cancer is the ninth most common cancer but the fifth leading cause of cancer death for women. It has long been known that ovarian cancer growth and development is dependent upon estrogen. Conversely, there is less known about how environmental estrogens influence ovarian cancer development. Estrogenic compounds have been shown to affect the epigenome, yet little is known about the specific mechanisms by which these changes occur. This project studied the effect of Bisphenol A (BPA) and Estradiol on expression of histone modifying enzymes (HMEs) in the SKOV3 and OVCAR3 human ovarian cancer cell models. This study compared the effects of BPA and Estradiol on Set8 (HMT) and Sirt1 (HDAC) expression under physiological conditions. Experiments were conducted

using Estradiol or BPA alone, or in combination with ICI, the estrogen receptor antagonist. . We found differences in the expression pattern of these genes between the two compounds and between the two model systems. These results imply that environmental estrogens potentially influence global gene expression by changing the expression of HMEs. Furthermore, these changes are different depending on the model system analyzed demonstrating a complex effect of these compounds in human ovarian cancer.

P1930

Board Number: B441

The effect of the estrogenic compounds E2 and BPA on the expression of histone modifying enzymes in prostate cancer cell lines.

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Prostate cancer continues to be the most commonly diagnosed cancer in males and it is known that exposure to estrogenic compounds can increase the likelihood of developing prostate cancer. The focus of this project was to determine the effect of physiological measurements of two estrogenic compounds, estradiol (E2) and bisphenol A (BPA) on certain histone modifying enzymes (HMEs) in prostate cancer cells. BPA is a known environmental estrogen that has the ability to bind to the estrogen receptor and induce biological activity classifying it as an endocrine disruptor. When activated, the estrogen receptor acts as a nuclear hormone receptor inducing transcription of target genes. The two HMEs studied were SET8, a histone methyltransferase (HMT) and SIRT1, a histone deacetylase (HDAC). Epigenetic regulation has been determined to be an important process in the maintenance of healthy cells and loss of this regulation has been linked to the development and progression of many cancers, including prostate cancer. To determine the role of these estrogenic compounds, LNCaP and PC3 prostate cancer cells were treated with various physiological doses of E2 or BPA alone and in conjunction with ICI, an estrogen receptor antagonist, or Casodex, an androgen receptor inhibitor. Cells were collected 24 hours post-treatment and RNA was isolated and purified. Isolated mRNA was analyzed and used to measure changes in the expression of SET8 and SIRT1 using endpoint PCR. The results of our experiments show that the expression of the two HMEs studied were differentially expressed by E2 or BPA alone as well in combination with ICI. There were distinct changes in expression between the two models as well. We have also demonstrated that expression of SET8 was not altered by treatment with E2 and Casodex, however, expression of SIRT1 was modified under these treatment conditions.

P1931**Board Number: B442****Identifying APC-dependent protein networks in *Drosophila melanogaster* using a complementary genetic and proteomic approach.**M.A. Blundon¹, D.R. Schlesinger¹, A. Parthasarathy¹, E. Kunttas-Tatli¹, B.M. McCartney¹, J.S. Minden¹;¹Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA

Wnt signaling is necessary for differentiation in all embryos, from flies to humans. Inappropriate Wnt pathway activation has been linked to a myriad of cancers, most notably breast and colorectal, as well as insulin insensitivity contributing to type II diabetes. The *Adenomatous polyposis coli* (*APC*) tumor suppressor encodes a multifunctional protein that is an essential negative regulator of Wnt signaling by contributing to β -catenin degradation through the destruction complex. In addition, APC functions in many cytoskeletal processes including actin assembly and microtubule network formation. Mutations in *APC* are found in 80% of all sporadic and hereditary colorectal cancers and disruption of APC's Wnt signaling function contributes to colon cancer initiation. While progress has been made in defining the normal functions of APC, controversy and significant gaps in our understanding remain.

We used *Drosophila* to identify novel APC-associated protein networks to more completely define APC's molecular role. APC proteins are conserved between humans and fruit flies, and much of APC biology elucidated in *Drosophila* has translated well to mammals. To understand the comprehensive consequences of loss of APC activity to protein networks, we took a complementary strategy coupling genetics and a proteomic technique called two dimensional difference gel electrophoresis (2D-DIGE). We used 2D-DIGE to detect visible proteomic changes between *wild type* and *APC* mutant embryos prior to the onset of significant zygotic transcription. We identified 16 obvious isoform changes corresponding to 17 proteins that are different between *APC2* null and *wild type* embryos. These isoform changes are likely the result of phosphorylation changes, suggesting that APC may facilitate a kinase or set of kinases that regulates these proteins. We prioritized the protein differences in three tiers by understanding the isoform changes under different mutant and rescue genetic conditions. The majority of the 9 prioritized proteins have been identified by mass spectrometry. Finally, our data support the hypothesis that many of the APC-dependent protein changes are also dependent on deactivation of the destruction complex and/or the accumulation of *Drosophila* β -catenin. Because our experiments are conducted at a time in embryogenesis prior to significant zygotic transcription, and the changes observed are exclusively isoform changes, we predict that these APC dependent protein differences are not the result of transcriptional activation of Wnt target genes. Taken together, this complementary genetic and proteomic approach has revealed novel APC-dependent proteomic changes that may contribute to colon cancer initiation and progression.

P1932**Board Number: B443****Regulation of mammalian pexophagy by oxygen-regulated hypoxia-inducible factors alpha.**M. Schönenberger¹, K.M. Walter¹, M. Horn¹, M. Trötz Müller², M.S. Lucas¹, H. Elsässer³, A.B. Moser⁴, T. Schwarz¹, P.A. Gerber⁵, P.L. Faust⁶, H. Moch⁵, H.C. Köfeler², W. Krek¹, W.J. Kovacs¹;¹ETH Zürich, Zürich, Switzerland, ²Medical University of Graz, Graz, Austria, ³Philipps-University Marburg, Marburg, Germany, ⁴Kennedy Krieger Institute, Baltimore, United States, ⁵University Hospital Zürich, Zürich, Switzerland, ⁶Columbia University, New York, United States

Peroxisomes play a central role in lipid metabolism and their function depends on molecular oxygen. Low oxygen tension or von Hippel-Lindau (*Vhl*) tumor suppressor loss is known to stabilize hypoxia-inducible factors alpha (Hif-1a and -2a) to mediate adaptive responses, but it remains unknown if peroxisome homeostasis and metabolism are interconnected to Hif-a signaling. By studying liver-specific *Vhl*, *Vhl/Hif1a*, and *Vhl/Hif2a* knockout mice we demonstrate a regulatory function of Hif-2a signaling on peroxisomes. Hif-2a activation augments peroxisome turnover by selective autophagy (pexophagy) and thereby changes lipid composition reminiscent of peroxisomal disorders. The autophagy receptor Nbr1 localizes to peroxisomes and is likewise degraded by Hif-2a-mediated pexophagy. Furthermore, we demonstrated that peroxisome abundance is reduced in *VHL*-deficient human clear cell renal cell carcinomas with high HIF-2a levels. These results establish Hif-2a as a negative regulator of peroxisome abundance and metabolism and suggest a mechanism by which cells attune peroxisomal function with oxygen availability.

P1933**Board Number: B444****Novel function of human Swi3 homologues BAF155 and BAF170 in aerobic respiration and tumor suppression.**M. Alam¹, J. Hooda¹, S. Lal¹, A. Shah¹, T.M. Cao¹;¹Molecular and Cell Biology, University of Texas at Dallas, Richardson, TX

The Swi3 human homologues BAF155 and BAF170 are known tumor suppressors. Previous studies have shown that they are essential components of the SWI/SNF chromatin remodeling complex. Importantly, emerging evidence strongly suggests that Swi3 and its human homologues can act independently of the Swi2/Brg1 to serve as a global regulator of aerobic respiration gene expression and oxygen consumption. An analysis of ChIP-Seq data showed that many genes involved in oxidative phosphorylation and mitochondrial respiration are regulated by both BAF155 and BAF170, but not Brg1. Further, in HeLa cells with BAF155 or BAF170 knocked down, the rate of oxygen consumption was significantly increased. Similarly, in yeast, deletion of the SWI3 gene, but not SWI2, caused a dramatic increase in oxygen consumption rate. Additionally, we found that Swi3 nuclear localization, but not Swi2

nuclear localization, requires oxygen. Further analysis showed that the transcription of genes encoding functions involved in oxidative phosphorylation was selectively increased in Δ swi3 cells. Likewise, measurements of mitochondrial respiratory chain complexes showed that their levels were increased in Δ swi3 cells. These results show that Swi3 and its human homologues BAF155 and BAF170 play a central role in the regulation of aerobic respiration genes and cellular bioenergetics. Inhibiting the function of Swi3 and BAF155 and BAF170 would lead to increased aerobic respiration and cellular energy generation. These results reveal a novel function of Swi3 and its human homologues and provide a molecular basis for the tumor suppressing function of BAF155 and BAF170. Experiments are currently underway to further ascertain the function of BAF155 and BAF170 in cellular respiration and tumor suppression.

P1934

Board Number: B445

The Rb tumor suppressor restricts reprogramming by directly silencing pluripotency networks.

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Reprogramming of differentiated cells to induced pluripotent stem (iPS) cells is a fascinating yet incompletely understood process. Based on the similarities between reprogramming and cancer, we investigated the tumor suppressor Rb in the induction of pluripotency. Here, we show that loss of Rb increases the efficiency of iPS cell reprogramming. Surprisingly, Rb inactivation does not enhance reprogramming by accelerating the cell cycle. Instead, Rb directly binds regulatory regions of many pluripotency genes, including *Sox2* and *Oct4*, and controls the chromatin state of these factors and their targets. Consequently, loss of Rb leads to a slight but widespread de-repression of the pluripotency program, rendering Rb-deficient cells reprogrammable without exogenous *Sox2*. Moreover, *Sox2* was found to critically mediate Rb-induced tumorigenesis. These results identify Rb as a global transcriptional repressor of the pluripotency network, explaining previous reports about Rb's involvement in cell fate pliability. Our observations also implicate factors involved in pluripotency such as *Sox2* in cancers driven by loss of Rb.

P1935**Board Number: B446****Epigenetic downregulation of *SPINT2* in aneuploidy.**H. Zhang¹, H. Li¹, N. Ganem², D. Pellman¹;¹Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA, ²Cancer Center, Boston University School of Medicine, Boston, MA

One aspect of genome instability – a hallmark of cancer – is increased rates of chromosome missegregation. Chromosome missegregation leads to aneuploidy, and consequently, the majority of tumors are aneuploid. While whole chromosome aneuploidy is known to impose a significant cost to cellular fitness, post-selection recurrent aneuploidy is thought to confer considerable evolutionary advantages in the context of tumorigenesis. It has been established that the proliferation of aneuploid mammalian cells is limited by a p53-dependent cell cycle arrest. We have identified the epigenetic downregulation of *SPINT2*, a putative tumor suppressor, to be an adaptive mechanism that allows aneuploid cells to bypass the p53-dependent arrest. The degree of *SPINT2* downregulation correlates with the severity of aneuploidy in hTERT RPE-1 cells. Additionally, *SPINT2* downregulation in aneuploid cells is associated with promoter hypermethylation, and its expression correlates with promoter CpG methylation density. Furthermore, depletion of *SPINT2* by RNAi in HCT116 colorectal carcinoma cells leads to proliferating, persistent aneuploid populations after experimentally induced chromosome missegregation. Our results indicate that disruption of *SPINT2* function renders mammalian cells more tolerant of aneuploidy. We aim to elucidate whether *SPINT2* mediates a tumor-suppressing mechanism that is prone to epigenetic misregulation and to understand the role of *SPINT2* in aneuploidy and tumorigenesis.

P1936**Board Number: B447****TM40, a novel target of p53, interacts with p53 and modulates cellular localization and function of p53.**S. Namkoong¹, K. Lee¹, R. Park², J. Park¹;¹Division of Biological science and technology, Yonsei University, Wonju, Korea, ²Yonsei University, Wonju, Korea

The p53 is one of the most important tumor suppressors and its malfunction has been reported in many types of cancer. Normally, the transcription level of p53 targets are decreased in the most common cancers. Here, we report that TM40 is the novel of transcriptional target of p53 and modulates the localization and transcriptional activity of p53. Microarray analysis revealed that TM40 expression is generally decreased in ovarian tumor and western blot analysis showed that depletion of p53 decreased the TM40 expression. Next, we examined the function of TM40 and found that TM40 is involved in transcriptional activity of p53. TM40 expression affects p53 phosphorylation as well as p53 localization. In addition, TM40 interacts with p53 and interferes with p53 dependent transcription. These results

collectively indicate that TM40, a target of p53, regulates the transcriptional activity of p53 by negative feedback mechanism.

P1937

Board Number: B448

Function of TBP-Like Protein (TLP) in p53-Governed Cell Growth Regulation.

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p53 is inactivated in about 50% of all human cancers, and induces growth arrest, DNA repair, or apoptosis in response to cellular stress such as DNA damage. Since p53 inhibits tumorigenesis, p53 is regarded as “the guardian of the genome”. It is well known that p53 interacts with many kinds of proteins and activates its target genes, though, overview of proteins that interact with p53 and their functions are remained unclear. TBP-like protein (TLP), which belongs to the TBP family proteins, regulates the expression of many genes involved in development and cell growth regulation. Previous studies show that TLP interacts with p53 and activates p21 gene expression in a p53-dependent manner, though detail of their molecular mechanisms is unknown. In this study, we investigated the role of TLP in regulation of p53 function. We found that TLP binds to the N-terminal region of p53 and activates p21 transcription in a p53-dependent manner. These observations suggest that TLP acts as a coactivator of p53. TLP over-expression and knock-down resulted in an increase and decrease of p53 protein level, respectively. Because TLP did not affect the mRNA level of p53, we speculated that TLP regulates p53 at protein level. Because p53 is down-regulated by MDM2 through polyubiquitination, we measured the amount of ubiquitin-conjugated p53. As expected, TLP over-expression decreased ubiquitin-conjugated p53. It is known that knock-down of TLP accelerates cell growth and inhibits damage-induced apoptosis. Consequently, it is suggested that TLP potentiates p53 function through stabilizing p53 protein, which subsequently modulates cell growth profiles and apoptosis induction.

Gene Structure and Transcription

P1938

Board Number: B450

Mammalian Glutamine Metabolism Controls Circadian Rhythm through Regulation of Reactive Oxygen Species.

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Circadian rhythms are twenty-four hour physiologic cycles present in all eukaryotes that control a variety of organismal processes, including metabolism. Peripheral clocks such as those present in the liver control metabolic pathways such as glucose metabolism and respiration as well as amino acid metabolism. It has been recently demonstrated that the availability of the metabolite NAD (nicotinamide adenine dinucleotide) can feed back to control circadian rhythm. There is much interest in targeting glutamine metabolism in cancer, but it is still unknown how inhibition of glutamine metabolism affects normal cell physiology, including circadian rhythm. Here we show using U2OS osteosarcoma cells, a commonly-used model of molecular circadian rhythm, that glutamine withdrawal blocked proper circadian oscillation of gene expression. Glutamine withdrawal led to distinct and dramatic changes in circadian gene expression which could be rescued by addition of the cell permeable TCA-intermediate α -ketoglutarate. However, cells withdrawn from glutamine did not show signs metabolic stress or impairment of the mTOR pathway. While alterations to histone modifications possibly stemming from impairment of α KG-dependent enzymes were observed, these did not explain the observed alterations in circadian rhythm. Rather, glutamine withdrawal led to the strong downregulation of several genes involved in reactive oxygen species (ROS) defense and neutralization. Further supporting the importance of ROS in regulation of circadian rhythm, addition of cell permeable antioxidants rescued the disruption of circadian oscillation in the absence of glutamine. Together, these data suggest that glutamine availability and metabolism are critical to support circadian rhythm and gene expression through modulation of intracellular ROS.

P1939**Board Number: B451****Transcriptome Analysis of Psoriatic Epidermis.**

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Split thickness skin grafts (STSG), consisting mainly of the epidermis and only a small amount of dermis, were taken from 9 healthy control (C), 5 psoriatic non-lesional (PN), and 6 lesional (PL) patients and subjected to RNA 5'-end sequencing. Total RNA per cell in PL was higher than in the C and PN samples; the data, therefore, was analyzed by using RNA spike-in normalization instead of global normalization. We found only 35 differentially expressed transcripts (DETs) in the PN skin when compared with the controls (FDR < 0.05). 28 of these were up-regulated (FC > 1.5) and only 7 down-regulated (FC < 0.75). Interestingly, 12 of the DETs map to the known *PSORS* loci, of which *PSORS4* was the most represented among the up-regulated ones. 2437 and 3541 transcripts were up-regulated and 2550 and 491 down-regulated in the PL, when compared with the C and PN samples, respectively. Gene set enrichment analysis with the differentially expressed genes (DEG) in PL, revealed enrichment at the *PSORS4* locus. The functional annotation analysis of DEGs highlighted gene ontology groups related to keratinocyte and epidermal cell differentiation, already in the PN samples. Furthermore, PL-DEGs showed enrichment in many of the functional groups previously associated with psoriasis: e.g. innate immunity and cell adhesion. RNA-Seq also enables the identification of different start sites and transcripts from the introns and untranslated regions. Within Top20 up-regulated transcripts from introns and untranslated regions, two are located at the *PSORS4* locus and therefore further highlighting the importance of the region. Comparison of our STSG DEGs with the previous gene expression data from full thickness (FT) psoriatic samples shows considerably less down-regulation, which may be due to the high amount of dermis in the FT samples; the expansion of epidermis in psoriasis leads to decrease in dermal genes. These results indicate that our samples enable direct focusing mainly on the epidermal transcripts. In addition, the sensibility allows a more concrete recognition of the altered signaling pathways; we identified much more altered components in each pathway than previous microarray studies, making it possible to get a better overall understanding about affected pathways and the network between them.

P1940**Board Number: B452****Mitochondrial DNA deletion breakpoints are strongly enriched for G-quadruplex-forming and direct repeat sequences.**B.A. Kaufman¹, D.W. Dong^{1,2};¹Department of Animal Biology School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, ²Penn Genome Frontiers Institute, University of Pennsylvania, Philadelphia, PA

Mitochondrial DNA (mtDNA) deletions are associated with the aging process and are a major cause of human mtDNA-related diseases. The sequence distribution of mtDNA deletion breakpoints around this circular genome is non-random. Although various mechanisms have been proposed recently, the role of sequence in deletion genesis remains inconclusive. In this study, we used novel and rigorous statistical measures to identify which of the proposed mtDNA sequence motifs are associated with mtDNA deletions.

To that end, we generated lists of six types of mtDNA motifs, including: G-quadruplex (GQ) forming sequences, stem-loop/cruciform (SC) structures, and several repeat sequences (direct, inverted, complementary, and inverted complementary). Those motif lists were then compared against the lists of mtDNA deletions found in healthy tissues or tissues of six clinical categories of diseases. For the full set of mtDNA deletions and for each disease category, we calculated the percentage of mtDNA deletions whose ends lie within 20 nucleic acid bases of either a repeat sequence pair (for each type of repeat motif), or two motif locations (for GQ or SC motifs). These values were compared to those calculated for controls, which were generated by rotating the corresponding motif set to every location along the circular human mtDNA to generate a finite p-value.

In the full mtDNA deletion set, a strong enrichment was detected for both ends being in close proximity to G-quadruplex motifs (p-values less than $10e-12$). Similarly, we found both ends of mtDNA deletions to be enriched near those of a direct repeat (p-values also less than $10e-12$). The mtDNA deletions enriched for these motifs preferentially showed enrichment at both breakpoints simultaneously. None of the other types of motifs produced enrichment of mtDNA deletions. Furthermore, we found that both G-quadruplex and direct repeat motifs were enriched in deletions from Parkinson's disease and healthy tissues. Direct repeat motifs also produced enrichment for tumor and inclusion body myositis. G-quadruplex motifs also produced enrichment for Kearns-Sayre syndrome, Pearsons syndrome, and progressive external ophthalmoplegia.

In conclusion, both G-quadruplex and direct repeats are associated with mtDNA deletions. For different diseases, different deletion formation mechanisms are potentially at work.

1941**Board Number: B453****Analysis of the Zct1 promoter, a key repressor of alkaloid production in *C. roseus*.**J.D. Weaver¹, E.J. Cram¹, C. Lee-Parsons²;¹Biology, Northeastern University, Boston, MA, ²Chemical Engineering; Chemistry and Chemical Biology, Northeastern University, Boston, MA

Because of their stationary existence, plants rely on swift, well-adapted responses to environmental stresses. Plants readily activate secondary metabolism and produce defense compounds in response to these stresses. *Catharanthus roseus* provides a relevant model for studying secondary defense pathways as it produces numerous Terpenoid Indole Alkaloids (TIAs) in response to certain environmental stressors, such as wounding or the presence of a pathogen. Furthermore, several TIAs produced are pharmaceutically valuable, most notably the anticancer drugs vincristine and vinblastine. However, these drugs are very expensive due to their low concentrations in the plant.

In the last two decades, there has been steady advancement in the understanding of TIA biosynthesis, including the sequencing of genes for the biosynthetic enzymes, discovery of transcription factors that regulate biosynthesis, transcriptomic analysis under various conditions, and elucidation of the pathway induced by the plant hormone Jasmonate (JA). JA has been shown to initiate TIA production by inducing expression of a network of transcription factors, including the repressor ZCT, and then expression of the biosynthetic genes. Here, we report the sequencing and analysis of the Zct1 promoter, a key transcriptional repressor of TIA biosynthesis. The full length Zct1 promoter and a series of deletions was used to drive expression of the GUS reporter gene in the Fast Agro-Mediated Seedling Transformation (FAST) method. The minimal promoter and relative importance of the identified elements was determined.

P1942**Board Number: B454****Molecular and functional characteristics of S-like ribonucleases from carnivorous plants.**N. Arai¹, E. Nishimura¹, S. Jumyo¹, K. Kuroda¹, T. Liu¹, T. Ohshima¹;¹Biology, Waseda University, Tokyo, Japan

Expression of S-like ribonucleases (RNases) is usually induced by phosphate starvation, wounding or senescence in plants. However, they are used for digestion of preys in carnivorous plants (Nishimura *et al.*, *Planta* 238, 955-967, 2013). To know molecular and functional characteristics of S-like ribonucleases from carnivorous plants, using the S-like RNases from *Drosera adelae* (sundew), *Dionaea muscipula* (Venus flytrap), *Cephalotus follicularis* (Albany pitcher plant), *Aldrovanda vesiculosa* (waterwheel plant), *Nepenthes bicalcarata* (fanged pitcher plant) and *Sarracenia leucophylla* (white pitcher plant) and those

from “non-carnivorous” plants, we recently performed a comparative study of S-like RNases. This study identified ten positions at which only carnivorous plants show the common usages of amino acid residues. Furthermore, it was revealed in a phylogenetic analysis that the S-like RNases of carnivorous plants form a group beyond the phylogenetic relationships of the plants (Nishimura *et al.*, *Planta* 240, 147-159, 2014). More recently, we have prepared the recombinant *D. adela* S-like RNase DA-I and its variants that have altered amino acid residue(s) at the above-described ten positions. These variants showed altered enzymatic properties, indicating the significance of the amino acid residues conserved among the carnivorous plants. The study of Nishimura *et al.* (2014) also suggested that the magnitude of kinetic parameters for carnivorous plant S-like RNases correlates negatively with the dependency on symbionts for prey digestion. To understand the structural background for this correlation, we are currently performing a detailed analysis on the structure-function relationship of the mutant enzymes.

P1943

Board Number: B455

Whole Exome Sequencing of Radiation Sensitive Patients Reveals Novel/Rare MCM2 Mutations.

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The relationship between ionizing radiation (IR) and human health is paradoxical; IR is a potent environmental carcinogen, but at the same time, it is one of the most effective therapeutic agents for the treatment of cancer. A greater understanding of how humans respond to IR exposure, both at the level of the whole organism and at the single cell level can inform the rational design of radioprotective or radiosensitizing agents. Many key features of the cellular and biochemical pathways that respond to IR damage have been elucidated through the study of rare autosomal recessive genetic disorders in humans characterized by hypersensitivity to IR. Prior studies of Ataxia-Telangiectasia (A-T) identified ATM as the critical signaling molecule in the DNA damage response (1), while similar studies of Nijmegen Breakage Syndrome (NBS) identified NBN and elucidated the role of the MRE11-RAD50-NBN (MRN) complex (2;3). Recently, the study of a single patient with RNF168 deficiency (RIDDLE Syndrome) revealed an important and unanticipated role for ubiquitination in the signaling of DNA double strand breaks (DSB) (4). We have studied the genetics of radiosensitivity in humans by focusing on recessive genetic disorders that result in the most extreme IR hypersensitivity phenotypes. During the course of these studies, we have accumulated cell lines from patients referred to us for diagnostic testing for A-T or NBS in whom no mutations in either the ATM or NBN genes could be found after exhaustive screening. These radiosensitive lymphoblastoid cell lines (RS-LCL) constitute a rich and unique resource for exploring and understanding the mechanisms of human cellular radiation hypersensitivity. In the present study, we applied exome sequencing to identify the causative mutations in our panel of RS-LCLs. Exome sequencing data have generated some candidate genes with novel and rare mutations that have not been previously reported; among these are a frameshift mutation in the exon 4 of minichromosome maintenance complex component2 (MCM2), which results in an early stop codon, and a missense

mutation in the exon 9 of MCM2 resulting in a replacement of one amino acid from glycine to arginine. Preliminary data show RS-LCLs harboring MCM2 mutations exhibit impaired colony formation and viability although the expression level of MCM2 mutant protein is comparable to wild type (WT) LCL. Mutant MCM2 protein is able to interact with other MCM members and forms complexes *ex vivo*. Further studies are necessary in order to elucidate the molecular mechanisms underlying the phenotypes of MCM2 mutant cell lines.

P1944

Board Number: B456

Alternative splicing of the PCLO gene is conserved between mice and zebrafish.

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Alternative splicing of genes is a fundamental process in multi-cellular organisms that ultimately leads to greater protein diversity. Conservation of alternative splicing events between orthologous genes of evolutionarily distant species has not been thoroughly examined. Here we investigated alternative splicing patterns between a mammalian species (*Mus musculus* or mouse) and a teleost species (*Danio rerio* or zebrafish) for the piccolo gene (*PCLO*). Piccolo is a multidomain protein with localized expression at presynaptic nerve terminals of neuronal synapses. Previous studies have revealed the presence of two primary alternative spliced transcripts of *PCLO* (a shorter isoform 2 and a longer isoform 1) in mouse brain. Isoform 1 contains two C2 calcium-binding domains (C2A and C2B) whereas the shorter isoform 2 contains only a single C2 domain (C2A). Further analysis of alternative spliced products of the mouse *PCLO* gene revealed additional minor splice variants including transcripts that lack the 27-nucleotide exon 16 found in the C2A domain-coding region. Deletion of exon 16 dramatically alters the calcium binding properties of the C2A domain. Recent studies have indicated that in zebrafish, a gene duplication event led to the generation of two versions of the Piccolo gene (*PCLO-a* and *PCLO-b*). Both genes exhibit striking similarity in genomic organization to their mouse and human homologs. Using RT-PCR analysis, we show that like mouse, both *PCLO-a* and *PCLO-b* from zebrafish can be generated into two primary alternative splice variants. Further analysis of alternatively spliced transcripts for both *PCLO-a* and *PCLO-b* revealed the presence of several minor splice variants including transcripts lacking the conserved 27-nucleotide exon in the C2A domain-coding region. Finally, using real-time PCR, we were able to quantify the gene expression of the two major splice variants in mouse and the four major splice variants in zebra fish. Our results indicate that in adult mouse brain, the longer isoform 1 is expressed at 3-fold higher levels than the shorter isoform 2. In zebrafish, the highest expressed transcript is the *PCLO-b* isoform 1 with this variant showing higher expression than the other three major splice variants (e.g. isoform 2 of *PCLO-b* and isoform 1 and 2 of *PCLO-a*). Our results provide evidence that for the *PCLO* gene both major and minor splicing events are conserved between two evolutionarily distant vertebrate species.

P1945**Board Number: B457****Utility of syntenic relationships of VDAC1 pseudogenes for not only an understanding of the phylogenetic divergence history, but also ascertaining possible pseudogene candidates as genuine pseudogenes.**Y. Ido^{1,2}, T. Yoshitomi^{1,2}, K. Ohkura³, T. Yamamoto^{1,2}, Y. Shinohara^{4,5};

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The voltage-dependent anion channel (VDAC), present in the outer mitochondrial membrane, is responsible for the free permeation of metabolites smaller than 5000 Da across this membrane; and 3 isoforms of VDAC, i.e., VDAC1, VDAC2, and VDAC3, are expressed in mammals. Our previous study on the transcripts encoding rat VDAC isoforms indicated the possible existence of an mRNA showing structural similarity with rat VDAC1. We assumed this unexpected mRNA would have been formed by transcription of this VDAC1 pseudogene, and so we explored pseudogenes of VDAC1 in the rat genome. As a result, we obtained 16 pseudogenes of rat VDAC1. The possible presence of pseudogenes of mouse and human VDAC was reported earlier, but their detailed characterization has not yet been achieved. To obtain a clue as to how and when pseudogenes of VDAC were formed in mammals, in this study we characterized pseudogenes of rat, mouse and human VDAC1. To identify candidates of pseudogenes of rat, mouse or human VDAC1, after having entered the amino acid sequence of rat, mouse or human VDAC1, the genome database of the target species was screened with tblastn program. As a result of this screening, we identified 16, 15 and 13 pseudogenes, respectively. When we loosened the screening conditions for pseudogenes we identified 4, 2 and 1 sequences, showing lower similarities with the VDAC1 sequence, and referred to them as "possible pseudogene candidates of VDAC1." None of the pseudogenes or pseudogene candidates identified in these genomes retained intron/exon boundaries of genuine genes encoding VDAC1, thus indicating that these sequences had been formed by retrotransposition of VDAC1 mRNA. The most intriguing question regarding studies on pseudogenes is how and when they are formed during the process of evolution. To answer this question, we further conducted synteny analysis of individual pseudogenes. No syntenic combination was observed with pseudogenes of VDAC1 between human and rodent, but 3 combinations were found to show synteny between mouse and rat. In this study, we discovered 3 syntenic pseudogene combinations of VDAC1 between mouse and rat genomes, which synteny was formed before their phylogenetic divergence. The obtained results are also expected to be useful for a better understanding of the molecular evolution of the VDAC genes.

P1946**Board Number: B458****Mechanisms of epigenetic silencing during hippocampal neuronal development.**L. Guajardo^{1,2}, R. Aguilar^{1,2}, B. Van Zundert¹, M. Montecino^{1,2};¹Center for Biomedical Research, Univ Andres Bello-Fac De Ciencias Biologicas, Santiago, Chile, ²FONDAP Center for Genome Regulation, Univ Andres Bello-Fac De Ciencias Biologicas, Santiago, Chile

During hippocampal neuron differentiation, the expression of critical inducers of non-neuronal cell lineages needs to be efficiently silent. Runx2 transcription factor is the master regulator of intramembranous osteoblast differentiation that leads to formation of the craniofacial bone tissue that surrounds and protects the brain in mammalian embryos. The molecular mechanisms that induce Runx2 gene silencing in brain cells and at the same time promote Runx2 expression in pre-osteoblastic precursors have not been explored. Here, we assess the epigenetic mechanisms that mediate osteoblast-specific gene silencing in neurons. In particular, we address the contribution of histone epigenetic marks, histone modifiers (Polycomb- and Trithorax-associated complexes), miRNAs and DNA methylation, on the expression of the Runx2/p57 bone-related isoform as well as its down-stream targets osterix and osteocalcin in hippocampal tissue obtained from rat embryos. Our results indicate that there is an enrichment of repressive chromatin histone marks and of the Polycomb PRC2 complex at the Runx2/p57 and osterix promoter regions. In addition, osterix and osteocalcin promoters exhibit extensive DNA (CpG) methylation. Moreover, we detect the presence of Runx2 mRNA-targeting miRNAs in hippocampal tissue. Silencing of Polycomb H3K27-methyltransferases Ezh2 and Ezh1, as well as overexpression of the Trithorax-subunit Wdr5, result in increased Runx2 mRNA expression. Additionally, we find that the expression of Ezh1 and Ezh2 changes during hippocampal neuron maturation and that a main mechanism to control this differential expression is mediated by miRNAs. Finally, we report that in immature neurons, expression of the Runx2/p57 protein re-activates transcription of early osteoblast-related genes and down-regulates expression of hippocampal genes. Together these results support a model where complementary epigenetic mechanisms efficiently silence osteoblastic genes during neuronal differentiation and at the same time promote the hippocampal phenotype. FONDAP15090007, FONDECYT3140418.

P1947**Board Number: B459****Isolation of Louisiana Crayfish RNA for gene expression investigation.**N. Gillette¹, A.A. Nagengast², I. Vatnick³;¹Biology, Widener University, Chester, PA, ²Biochemistry, Widener University, Chester, PA, ³Biology and Biochemistry, Widener University, Chester, PA

Aquatic ecosystems around the world are constantly polluted by the domestic and agricultural use of common herbicides such as Round-Up and Atrazine. Run-off causes leaching of these chemicals into aquatic ecosystems, exposing all organisms to their harmful effects. We are particularly interested in

understanding the physiological and molecular effects that herbicide exposure has on crayfish, a commercially important food product in the U.S. and in Argentina. Collaborative studies with colleagues at the University of Buenos Aires have shown that crayfish exposed to the active ingredients of herbicides like Round-Up and Atrazine exhibit reduced growth and a metabolic response. Our current extension of this project aims to identify the molecular cause of these affects. Understanding the molecular modification that may be occurring following exposure begins with an understanding of the genes that may be expressed or repressed following herbicide exposure. We started by determining an efficient method of RNA isolation from the hepatopancreas tissue of the Louisiana Crayfish. Following the comparison of two common RNA isolation techniques, we determined that the RNeasy mini prep kit, that utilizes Trizol, is a simpler and more efficient mode of isolating RNA from the Louisiana Crayfish. With viable RNA isolated, we proceeded to perform a microarray with *Drosophila* chips to identify the genes expressed in the hepatopancreas of untreated Louisiana Crayfish. However, abundant hybridization did not occur between isolated RNA and the fly chip. Therefore, we plan to reiterate this procedure and will optimize the isolation of Louisiana Crayfish RNA, so as to produce RNA of greater purity. If successful hybridization occurs when using a fly chip again, we will then utilize this microarray technique with isolated RNA from Louisiana Crayfish exposed to herbicides. This analysis will identify changes in gene expression that may occur following herbicide exposure.

P1948

Board Number: B460

Genetic regulation of the temperature-dependent egg-laying rate in *C. elegans*.

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In humans, muscle function depends on temperature, but due to genetic complexity, it is difficult to identify genes responsible for this dependence. The genomes of humans and *Caenorhabditis elegans* contain many homologues, enabling *C. elegans* research to provide a blueprint for studying temperature-dependent muscle function in humans. Muscle function can be measured quantitatively in *C. elegans* by tracking egg-laying, since when an egg is laid, the vulval muscles of the worm must contract. These muscles function at different rates across a range of temperatures, meaning that the worms' muscles contract more often at certain optimum temperatures than at others. We have found that the egg-laying rates and optimum egg-laying temperatures also differ among strains of *C. elegans*, despite the presence of only minor genetic differences. By comparing the egg-laying rates and genomic sequences of two phenotypically divergent strains, N2 (Bristol) and CB4856 (Hawaii), we have identified loci within chromosomes IV and X of the *C. elegans*' genome that may affect muscle function. After specific genes of interest are identified in *C. elegans*, homologous genes in humans can be studied for their roles in temperature dependence of muscle function.

P1949**Board Number: B461****The NuA4 and Swr1 chromatin modification complexes are important for RNA splicing.**D. Sprague¹, N. Paripati¹, A. Moazamian², R. Moazamian³, J. Martinez⁴, T.L. Kress¹;¹Biology, The College of New Jersey, Ewing, NJ, ²Biology, Celloxess LLC, Pennington, NJ, ³Celloxess LLC, Pennington, NJ, ⁴Thomas Jefferson Medical College, Philadelphia, PA

The ability to quickly and precisely regulate gene expression is a fundamental biological process of paramount importance to cell survival. Two important steps in gene expression are transcription and splicing. These steps are coordinated to ensure accuracy and efficiency, yet very few proteins that function in this coordination have been identified. Recent high throughput genetic interaction studies using *Saccharomyces cerevisiae* revealed that splicing factors interact with factors that are important for transcription. We have utilized a targeted genetic screen to identify novel interactions between splicing factors and factors that modify chromatin to modulate transcription. Using both qualitative and quantitative growth assays we identified negative genetic interactions between genes encoding splicing factors and both *SWR1*, a component of the Swr1 chromatin remodeling complex, and *HTZ1*, which encodes the variant histone inserted into chromatin by Swr1. In addition, our screen revealed novel negative genetic interactions between splicing factors and *EAF7*, a component of the NuA4 histone acetyltransferase complex. Notably, both the NuA4 and Swr1 complexes function together to regulate transcription. Using quantitative RT-PCR we have shown that mutation of individual components of the NuA4 or Swr1 complexes or Htz1 causes a modest block in RNA splicing and exacerbates the splicing defects observed in a yeast strain lacking a splicing factor, as predicted by our genetic analysis. Taken together, these data support a model in which the NuA4 and Swr1 chromatin modification complexes interact with the splicing machinery to coordinate transcription and splicing.

P1950**Board Number: B462****The Set2 histone methyltransferase is required for efficient RNA splicing.**S. Ucles¹, M. Sorenson², D. Flood¹, S.W. Stevens², T.L. Kress¹;¹Biology, The College of New Jersey, Ewing, NJ, ²University of Texas at Austin, Austin, TX

Proper gene expression involves multiple steps, including RNA splicing where non-protein coding regions of RNA are removed from the RNA transcript. RNA splicing is carried out by the large and dynamic spliceosome, which is comprised of small ribonucleoprotein complexes (snRNPs) that assemble on an RNA molecule in a precise order. Assembly of the spliceosome occurs co-transcriptionally and RNA splicing is tightly coordinated with transcription to ensure precise and efficient gene expression. However, the mechanisms that underlie this coordination are poorly understood. In order to identify proteins that function to coordinate RNA splicing with transcription we carried out a genetic interaction

study using *Saccharomyces cerevisiae*. We identified negative genetic interactions between genes encoding RNA splicing factors and the *SET2* gene, a histone methyltransferase that methylates lysine36 on histone H3 (H3K36) to regulate transcription. Furthermore, we show that mutations that block H3K36 methylation also have negative genetic interactions with splicing factor genes, suggesting that H3K36 methylation is important for RNA splicing. Indeed, we have shown that deletion of *SET2* or point mutation of H3K36 inhibits RNA splicing and exacerbates splicing defects in yeast strains harboring deletions of splicing factor genes. Using chromatin immunoprecipitation, we demonstrate that deletion of *SET2* reduces the association of snRNPs with chromatin. Thus, we provide the first evidence that H3K36 methylation is required for appropriate RNA splicing in yeast and suggest a model in which Set2 or H3K36 methylation help to recruit splicing factors to RNA during transcription.

P1951

Board Number: B463

Mio acts in the insulin-producing cells of the *Drosophila* brain to control feeding and metabolism.

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During times of nutrient abundance, animals recognize the availability of nutrients and regulate their intake and storage of these nutrients accordingly. However, the molecular mechanisms underlying nutrient sensing and subsequent changes in behavior and metabolism are not fully understood. The production and secretion of the peptide hormone insulin has been shown to be nutrient responsive and affect metabolism in many different organisms ranging from flies and worms to mice and humans. Our lab has recently shown that *Mio*, the *Drosophila* homolog of carbohydrate response element binding protein (ChREBP), functions in the fat body (the equivalent of liver and adipose tissue of the fly) to control triglyceride storage as well as feeding suggesting that *Mio* may act as a nutrient sensor to coordinate food consumption and metabolic homeostasis. Here, we demonstrate that *Mio* functions in the insulin-producing cells (IPCs) of the fly to regulate feeding and metabolism. Targeted disruption of *Mio* specifically in the IPCs inhibits feeding and alters nutrient storage. Interestingly, *Mio* may be acting in the IPCs by controlling the expression of hormones secreted from these cells as IPC-specific RNAi of *Mio* leads to a decrease in *Drosophila insulin-like peptide (dilp) 2* and an increase in *dilp3* levels. Furthermore, disruption of *Mio* function in the IPCs does not affect *dilp5* or *Drosulfakinin (Dsk* — the *Drosophila* homolog of mammalian *cholecystokinin*) expression. These data indicate that *Mio* functions as a nutrient sensor in the IPCs by controlling insulin-like peptide expression leading to the regulation of feeding and metabolism in accordance with nutrient availability.

P1952**Board Number: B464****Epigenome-wide evaluation of short-term DNA methylation stability in monocytes.**R. Furukawa¹, T. Hachiya¹, H. Ohmomo², Y. Shiwa¹, K. Ono², S. Suzuki³, M. Satoh¹, J. Hitomi⁴, K. Sobue⁵, A. Shimizu²;

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Cytosine methylation in CpG dinucleotides is one of the key epigenetic mechanisms that regulate gene expression. DNA methylation is considered to be a stable, and to define "fields" for producing the transcriptomes that characterize the functional differences of cells and tissues. On the other hand, gene expression patterns are well known to change in response to various stimuli in the body. It remains unclear to what extent short-term dynamics in gene expression involves epigenetic mechanisms, especially DNA methylation. In the present study, we examined the short-time dynamics and stability of gene expression and DNA methylation of monocytes that show relatively low heterogeneity in blood cells. We collected blood 24 times from two healthy volunteers (males in thirties) in three months, obtained CD14^{high}CD16^{low} monocytes using cell sorter (SH800, Sony), and extracted DNA and RNA. Methylation levels of approximately 480,000 CpG probes were measured by Illumina Infinium HumanMethylation450 BeadChip microarrays, and were evaluated by β -values, which are the ratio of the methylated probe intensity and the total signal intensity (ranged from 0 to 100 %). For >97% of CpG probes, the maximum differences of DNA methylation levels between the 24 times were less than 10%. On the other hand, the maximum differences of DNA methylation levels exceed 50% at 81 CpG probes. Next, in order to investigate whether these variations of DNA methylation level affect expression of neighboring genes, we conducted RNA sequencing, and then detected Methylation-Expression associations (MEAs) using a linear regression model and the ANOVA test. Of 341,233 CpG positions located on the promoter or gene body regions, 2,819 (0.83%) were associated with the expression of neighboring genes with a significance level of 0.01. In these MEA CpG positions, the maximum differences of DNA methylation levels exceed 50% at only one CpG probes, and in approximately 96% of the genes detected by MEA analysis, the maximum difference of the $\log_{10}[\text{FPKM}+1]$ were less than 0.3. From these results, the drastic variation of DNA methylation does not seem to contribute much to the dynamic change of gene expression. However, a possibility that small or moderate changes of DNA methylation may affect the gene expression is required to be explored.

P1953**Board Number: B465****Expression Levels of DNA Damage Response Genes in Zebrafish (*Danio rerio*) following Treatment Methylnitrosourea, Doxorubicin, or Ultraviolet light.**J. Woolcock¹, H. Khan¹, K. Joseph¹, E.E. Gestl¹;¹Biology, West Chester Univ, West Chester, PA

While much of the signaling of DNA damage is coordinated through post-translational modification, the expression levels of five genes, ATM, p53, p21, chk1, and chk2 have been examined in zebrafish at different stages of life ranging from two hours to adults (males and females) three years of age. Zebrafish are vertebrate, freshwater fish that grow 3-4 cm in size and are relatively easy to maintain at low costs. Each pair of fish can produce hundreds of eggs biweekly with a generation time of 3 months. The embryos develop ex vivo and are completely transparent during the first week of life, allowing for the observation of internal structures. This study utilized RNA collected from both untreated zebrafish embryos, and embryos treated with Ultraviolet light, N-nitroso-N-methylurea (MNU), or Doxorubicin. The level of mRNA expression from these genes at each stage was determined using quantitative reverse transcriptase polymerase chain reaction (q-RT PCR) and SYBER Green in relation to actin. Both ATM and p21 exhibited high levels of gene expression within untreated samples isolated from older zebrafish such as 3 years of age. Every gene treated with MNU and Doxorubicin increased in expression as the concentration was raised, however, ATM was the only gene that showed an increase in its expression following exposure to ultraviolet radiation. The expression of DNA damage response genes examined in zebrafish will further establish this vertebrate as a model organism to study DNA repair mechanisms.

P1954**Board Number: B466****Evolution of novel response element specificity in the glucocorticoid receptor.**W.H. Hudson¹, E.A. Ortlund¹;¹Department of Biochemistry, Emory University, Atlanta, GA

Specific recognition of DNA response elements by transcription factors is a key step in the regulation of gene expression. Some transcription factors recognize multiple, distinct response elements to mediate transrepression or transactivation of target genes. However, the mechanism by which a single transcription factor can evolve to recognize multiple response elements is unclear. Here, we study the evolution of the glucocorticoid receptor, which recognizes distinct response elements to mediate activation or repression its target genes. The glucocorticoid receptor binds to activating glucocorticoid response elements, or (+)GREs, as a dimer to activate transcription. Alternatively, GR binds to negative glucocorticoid response elements (nGREs) in a monomeric fashion to repress transcription. We demonstrate that, of the extant 3-keto steroid receptors, only the glucocorticoid receptor can bind to and repress transcription in a nGRE-dependent manner, despite the ability of all 3-keto steroid receptors

to bind to and activate gene transcription from (+)GREs. Surprisingly, using ancestral gene reconstruction, we find that nGRE binding and gene repression was a feature of the ancestor of all extant 3-keto steroid receptors. nGRE binding and repression originated as a subfunction of the ancestral 3-keto steroid receptor coincident with (+)GRE binding, and this subfunction was optimized in the evolutionary lineage of the glucocorticoid receptor but lost in the ancestors of the mineralocorticoid, progesterone, and androgen receptors. Further, using x-ray crystallography and other structural biology approaches, we define the structural mechanisms by which the modern-day DNA binding specificity evolved in the glucocorticoid receptor.

P1955

Board Number: B467

Determination of Expression Levels of all 20 DNA Polymerases in Zebrafish (*Danio rerio*) throughout Development and following Treatment with MNU, Doxorubicin, and UV Radiation.

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Zebrafish like humans contain sixteen DNA polymerases encoded by 20 different genes, the most recent uncovered in 2012. The expression of the DNA polymerases were determined at stages of development throughout the zebrafish (*Danio rerio*) life establishing it as an excellent model for the study of basic biological processes including DNA replication. RNA isolated from zebrafish of varying ages from 2 hours to 3 years old was used for Reverse-Transcriptase PCR to determine the level of polymerase gene expression throughout zebrafish development. All the polymerases were expressed at every stage of development examined. Quantitative RT-PCR was used to determine the relative quantity of polymerase expression by comparison with actin. While polymerase replicative polymerases like polymerase delta1 and delta2 resulted in similar levels in all the developmental stages, polymerases iota and eta were detected at decreased levels, most possibly due to their specialized roles in DNA replication. The expression level of polymerases beta and eta was highest in older stages of zebrafish development. The polymerase expression levels were also examined following exposure of 1-day old zebrafish embryos to the doxorubicin and methylnitrosourea, or ultraviolet radiation. One of the primary results was an increase in the level of polymerase eta expression due to its role in thymine-thymine photoproduct bypass. This completed study of polymerase expression results in a comprehensive model of DNA replication in zebrafish that can be utilized in future research.

P1956**Board Number: B468****GcrA is a global transcriptional regulator promoting open complex formation at methylated promoters in *Caulobacter*.**D.L. Baer^{1,2}, M.T. Laub^{1,2};¹Biology, Massachusetts Institute of Technology, Cambridge, MA, ²Howard Hughes Medical Institute, Cambridge, MA

Global regulators, unlike classical bacterial transcription factors, associate with RNA polymerase (RNAP) prior to binding DNA, and are found at promoters in a sequence non-specific manner. However, the extent of their effect on gene expression and mechanism of action are incompletely defined. Here, we show that GcrA, a major regulator of cell cycle progression in *Caulobacter crescentus* is a global regulator. GcrA forms a stable complex with RNAP holoenzyme through an interaction with σ^{70} , the primary sigma factor. Consistent with this interaction, ChIP-Seq analysis reveals that GcrA is present at nearly all σ^{70} -dependent promoters. Surprisingly, although GcrA is found at σ^{70} -regulated promoters in a sequence non-specific manner, only promoters harboring an extended recognition element that includes a subset of GANTC methylation sites are affected by GcrA. GcrA promotes open complex formation at these methylated promoters by interacting with Region 2 of σ^{70} , the region involved in binding the -10 element during strand separation. Guided by these mechanistic studies, we combine ChIP-Seq and expression profiling to pinpoint the direct GcrA regulon. Our work elucidates a new mechanism for transcriptional regulation and shows how this regulation contributes to a reprogramming of gene expression during a key stage of the cell cycle in *Caulobacter*.

P1957**Board Number: B469****Transcription regulation of LRRK2 by Egr-1 in B lymphocyte.**H. Han¹, H. Li¹, Y. Zou¹, Z. Zhang²;¹Central South University, State Key Laboratory of Medical Genetics, Changsha, China, ² Central South University, State Key Laboratory of Medical Genetics, Changsha, China

The leucine-rich repeat kinase 2 (LRRK2) mutations are the most common genetic cause of autosomal-dominant Parkinson disease (PD) and is also associated with susceptibility to Crohn's disease and leprosy. Currently, pathogenesis of PD caused by the LRRK2 mutations are poorly understood, and the physiological activities of LRRK2 as well as its transcription regulation are still elusive. Here we characterized the 5'-flanking region of the human LRRK2 gene and identified Egr-1 as a transcription regulator of LRRK2, the nine nucleotides GCGTGGGCG located in the 5'-flanking region of the human LRRK2 gene constitute the Egr-1 binding site. Knockdown endogenous Egr-1 reduces LRRK2 transcription in Epstein-Barr virus transformed B lymphocytes. Phorbol 12-myristate 13-acetate (PMA), which caused a rapid induction of Egr-1, can induce LRRK2 gene expression. LRRK2 modulates IL-6 production in PMA activated Epstein-Barr virus transformed B lymphocytes, indicating that LRRK2 takes part in cytokines

production in activated B cells. Moreover, Egr-1/LRRK2 pathway is critical for constitutive Immunoglobulin G(IgG) secretion. These data suggest that LRRK2 regulates immune responses through mediating B cell function and help us to understand the pathophysiological function of LRRK2 in the immune system.

Nucleocytoplasmic Transport 2

P1958

Board Number: B471

Nucleocytoplasmic Partitioning and Dynamics of a Vertebrate Proteome.

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Despite the nucleus' central role in multi-cellular biology, how the cell's proteome is partitioned between nucleus and cytoplasm is still poorly understood. This is mostly due to the difficulty of separating nuclear and cytoplasmic content and the challenge to comprehensively measure relative protein abundance and dynamics. Here, we quantify the nucleocytoplasmic distribution for more than 9000 proteins, with two different methods of quantitative proteomics, using the giant *Xenopus laevis* oocytes, which allow nuclear isolation via microdissection. We find a trimodal distribution, where most proteins localize exclusively to the nucleus or the cytoplasm, while a third subset of proteins is nearly equidistributed. By measuring the physiological protein size in undiluted cytoplasm we show that nearly all partitioned proteins have a physiological size larger than ~100kDa, while physiologically smaller proteins are typically equipartitioned. This suggests that protein assembly plays an important and so far underappreciated role in proteins' retention within a membrane bound organelle. Unexpectedly, we also find multiple examples of large, equipartitioned complexes. By following subcellular protein dynamics upon nuclear export perturbation with Leptomycin B, we provide evidence that these complexes are equipartitioned by active bi-directional nuclear transport. Surprisingly, this perturbation also re-localizes many kinases towards the nucleus, suggesting an intriguing explanation for the efficacy of this drug class in the treatment of various cancers. Thus, we present the first resource for the quantitative nucleocytoplasmic partitioning of a vertebrate proteome, measure its dynamics upon perturbation, shed new light on the mechanisms of subcellular protein localization, and suggest novel mechanisms of action for a cancer therapeutic.

P1959**Board Number: B472****Single Molecule Real-Time (SMRT) Imaging of HIV-1 RNA Modulation by DDX1 in Living Cell.**L. Tu¹, A. Joseph¹, H. Chu², L. Gerace², D. Grunwald¹;¹RNA Therapeutics Institute, University of Massachusetts Medical School, Worcester, MA, ²Department of Cell and Molecular Biology, Scripps Research Institute, La Jolla, CA

Evidence is building that DDX1, a DEAD-box helicase, participates in rev-assisted HIV-1 RNA genome transport processes. We are particularly interested in the spatial and temporal landscape of this rev-RRE and DDX1 interaction in the cell nucleus as well as the binding states. Specifically we aim to understand the life cycle of the HIV-1 genome, beginning at the transcription site of the integrated pro-virus, during intra-nuclear “transport” of the HIV-1 genome (i.e. diffusion to the nuclear periphery) to its appearance in the cytoplasm.

To study HIV-1 RNA and DDX1 interactions in real time in the living cell we engineered an optimized fluorescent cell line which can be used in single molecule microscopy techniques with nanometer and millisecond resolution. This cell line is based on a previously described U2OS derived cell line that harbors 75 stably integrated copies of replication deficient HIV genome labeled using a 24x MS2 cassette (Boireau et al. J Cell Biol. 2007). After selection of a subclonal population of cells that express replication deficient HIV genome, we stably integrated a nuclear pore marker (POM121), a MS2 coat protein, a codon optimized DDX1 and shRNA(s) against endogenous DDX1. All integrated sequences are fused to a fluorescent protein sequence except for the shRNA(s). Cell populations were selected either by fluorescence levels or via antibiotics in case of the shRNA(s). We show that the codon optimized DDX1 can rescue a full depletion of endogenous DDX1. This allows us to modify the amount of labeled, optimized DDX1 relative to endogenous DDX1 and further optimize our cell line for all three fluorescent tags to visualize and track HIV-1 RNA and DDX1 molecules in real-time in living cells by single molecule microscopy.

P1960**Board Number: B473****The mRNA export factor NXF1:NXT1 forms a symmetric binding platform for retroviral RNA export.**S. Aibara¹, E. Valkov¹, J. Katahira², M. Stewart¹;¹MRC Laboratory of Molecular Biology, Cambridge, United Kingdom, ²Graduate School of Frontier Biosciences, Osaka University, Osaka, Japan

The NXF1:NXT1 complex (also known as TAP:p15) is a general mRNA nuclear export factor with homologs from yeast to humans (1). NXF1 contains four domains: RRM (RNA recognition motif), LRR (leucine rich repeat), NTF2L (nuclear transport factor 2-like), and UBA (ubiquitin associated domain) and

forms a tight heterodimeric complex with NXT1 through the NTF2L domain (2). NXF1:NXT1 is thought to facilitate mRNA export using direct interactions with both mRNA and nuclear pore proteins (2).

NXF1:NXT1 also facilitates the rapid nuclear export of unspliced retroviral genomic RNA from simple type-D retroviruses, such as simian betaretrovirus serotype 1 (SRV-1), that contain a constitutive transport element (CTE), a *cis*-acting two-fold symmetric RNA stemloop motif. NXF1:NXT1 mediates the export of CTE-tagged transcripts more efficiently than other transcripts and can also block the export of native mRNAs (3-5). The principal regions of NXF1 involved in RNA binding have been thought to be the RRM and LRR domains and recently a crystal structure has been obtained of them bound to half of the CTE RNA (6) motif. However, how NXF1:NXT1 binds to complete CTE RNA and whether this involves any higher organization of the NXF1 domains remains unknown.

We have obtained a 3.4 Å crystal structure of the LRR and NTF2L domains of human NXF1 complexed with NXT1. Two copies of this complex were present in the asymmetric unit and were arranged to form a domain-swapped dimer, in which the linkers between the LRR and NTF2L domains of each complex formed intimate interactions with the NXT1 of the other complex, generating a two-fold symmetric platform. We have identified residues in this linker and engineered mutants that demonstrate dimer formation is important for the CTE-mediated enhancement of export of CTE-tagged transcripts. Combining complementary structural, biochemical, and ex vivo data, we have developed a plausible model for the recognition of CTE RNA by a symmetric RNA binding platform formed by a domain-swapped dimeric NXF1:NXT1 complex.

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1. Katahira, J., et al. (1999) EMBO J. 18:2593–609.
2. Valkov, E., et al. (2012) Biochim. Biophys. Acta, 1819:578–92.
3. Braun, I.C., et al. (2001) J. Biol. Chem. 276:20536–43.
4. Ernst, R.K., et al. (1997) RNA, 3:210–22.
5. Grüter, P., et al. (1998). Mol. Cell, 1:649–59.
6. Teplova, M., et al. (2011) Nature Struct. Mol. Biol., 18:990–8.

P1961

Board Number: B474

Intracellular trafficking of nuclear β -dystroglycan.

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β -dystroglycan (β -DG) is a transmembrane protein that belongs to the Dystrophin Associated Protein Complex (DAPC). β -DG has been widely described at the plasma membrane (PM), where it confers integrity to the phospholipid bilayer and participates as scaffold for signal transduction. Recently we described the presence of β -DG in the nucleus of different cell lines and characterized its nuclear import pathway, which is mediated by an importin α / β -recognized NLS, localized in the juxtamembrane region

of β -DG. The aim of the present study was to define the intracellular traffic that β -DG undergoes to reach the nucleus. Treatment of C2C12 cells with brefeldin A (BFA) to block endoplasmic reticulum (ER) to Golgi anterograde transport revealed that β -DG requires passing throughout Golgi to be further localized to the nucleus. Biotinylation of plasma membrane proteins and subsequent nuclei isolation showed that β -DG is translocated from PM to the nucleus. Inhibition of endocytic pathway using dynasore resulted in decreased nuclear localization of β -DG, which suggests that endocytic vesicle-dependent retrograde transport is involved in β -DG nuclear translocation. Identification of biotinylated β -DG in a purified endoplasmic reticulum fraction indicated that β -DG reaches this organelle before its nuclear translocation. Overall, our results suggest that β -DG releases from PM via endocytosis and transits through ER as part of its retrograde trafficking to the nucleus.

P1962

Board Number: B475

Molecular determinants of nuclear size in melanoma cancer cells.

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Pathologists assess changes in nuclear size and morphology to diagnose and stage various cancers. We hypothesize that increases in nuclear size contribute to metastatic capacity by promoting proliferation and/or invasive potential. Some factors that regulate nuclear size have been identified and experiments in *Xenopus* showed that nuclear import rates correlate with nuclear size. The levels of two nuclear import factors, importin α and Ntf2, were shown to be particularly important, modulating the import rate of nuclear lamins. The goal of our study is to quantify differences in nuclear size and the nucleocytoplasmic ratio between normal and cancer cell lines and to determine how the levels of known nuclear scaling factors vary during carcinogenesis. We utilize different stage melanoma cell lines, including primary melanoma (radial and vertical growth phases) and metastatic melanoma, as well as a normal melanocyte cell line as a control. We have quantified large differences in nuclear size between normal melanocytes and three different radial growth phase melanoma cell lines. We also observed a small but statistically significant difference in nuclear size between a vertical growth phase and metastatic melanoma cell line derived from the same patient. Surprisingly, the largest nuclear sizes were consistently observed in radial growth phase primary melanomas. To better understand this result, we are currently measuring nuclear-to-cytoplasmic volume ratios in these cell lines. We observed an inverse relationship between nuclear size and Ntf2 protein levels in whole cell extracts. Normal melanocytes with the smallest nuclei have the highest Ntf2 levels, while radial growth and metastatic melanomas with the largest nuclei have very low levels of Ntf2. These data were confirmed in cells by Ntf2 immunofluorescence staining. All these data suggest that Ntf2 may play a role in nuclear size changes during melanoma progression. To directly test this idea, we will manipulate the levels of Ntf2 in these various cell lines and observe how this impacts nuclear size. We plan to generate stable cell lines in which we can inducibly vary the levels of Ntf2, as well as other nuclear scaling factors. As a proof of principle, we find that knocking down the levels of importin alpha in metastatic melanoma cells leads to

a reduction in nuclear size. We will use our inducible cell lines to examine how altering nuclear size affects cancer cell characteristics such as invasive potential and rates of proliferation and apoptosis.

P1963

Board Number: B476

Nuclear localization of eEFSec and its role in Selenocysteine incorporation.

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Selenoproteins form the bulk of a cell's antioxidant defense system and contain the 21st amino acid, Selenocysteine (Sec). Glutathione peroxidases, thioredoxin reductases and deiodinases are selenoproteins that perform both housekeeping and stress-response functions in the cell. Sec is synthesized from the essential trace element selenium (Se) and is highly reactive. Thus, it is often present in the active site of the above enzymes. The translation of selenoprotein mRNAs itself has a unique mechanism. An in-frame UGA codon, that serves as a canonical termination codon, gets recoded to incorporate Sec. This specialized elongation cycle requires several dedicated and unique *cis* and *trans* acting factors, including the Sec-specific elongation factor called eEFSec. Unlike canonical translation elongation factor eEF1A, eEFSec binds only one species of tRNA -- the selenocystnylated-tRNA^{Sec}, and contains four domains instead of three. Previous studies have highlighted the importance of eEFSec Domain IV for all eEFSec functions during Sec incorporation. However, little is known about the subcellular localization, interacting partners and regulation of eEFSec. This study confirms the nucleo-cytoplasmic shuttling of eEFSec, which is rare for elongation factors as most are predominantly located in the cytoplasm. Additionally, specific residues that allow for nuclear localization as well as nuclear export of eEFSec have been identified. Our data suggest that eEFSec contains a bipartite nuclear localization signal, located in the C-terminus of the protein and a nuclear export signal located in the N-terminus. Moreover, these residues are critical for eEFSec to be able to support Sec incorporation *in vitro*, suggesting that nucleo-cytoplasmic shuttling of eEFSec may be critical to its function. Finally, immunoprecipitation experiments with eEFSec as the bait have identified two nuclear proteins involved in ribosomal biogenesis as eEFSec binding proteins. Thus, a model can be constructed where eEFSec is co-exported from the nucleus in a complex with ribosomal subunits, and that disruption of the residues involved in nuclear localization or export interfere with eEFSec's ability to interact with ribosomes during Sec incorporation. Future work includes determining the nuclear export pathway utilized by eEFSec, as well as the role and regulation of eEFSec localization during selenoprotein biosynthesis.

P1964**Board Number: B477****Beta-Adducin is Phosphorylated in The Nuclei of HeLa Cells by Protein Kinase C.**H.P. Pariser¹;¹Biology, Salem State Univ, Salem, MA

The 87 kDa protein adducin 2b is a cytoskeletal protein responsible for increasing the stability of actin-spectrin networks by behaving as an actin-filament-capping protein in the cytosol. The plasticity of the actin-spectrin network is regulated through the phosphorylation of serine 713 and 726 in the myristoylated alanine-rich protein kinase C (PKC) substrate domain. More recently it has been shown adducin 2b translocates to the nuclei of HeLa cells in response to the cytokine pleiotrophin (PTN) where it has been shown to be phosphorylated at serine 713 and 726. Adducin 2b also becomes associated with centrioles and condensed chromosomes in dividing cells. Here we demonstrate that adducin 2b is a substrate target for PKC when HeLa cell nuclear extract is treated with the PKC activator phorbol 12-myristate 13 acetate (PMA). This result suggests that adducin 2b is regulated in the nucleus and is possibly the mechanism by which adducin 2b associates with nuclear chromatin.

P1965**Board Number: B478****Elucidating mechanisms of nuclear size regulation using cell-free cytoplasmic extracts and microfluidic droplet-generating technology.**J.W. Hazel¹, K. Krutkramelis², P. Jevtic¹, D. Levy¹, J. Oakey², J. Gatlin^{1,3};¹Department of Molecular Biology, University of Wyoming, Laramie, WY, ²Department of Chemical and Petroleum Engineering, University of Wyoming, Laramie, WY, ³Molecular Cellular Life Sciences Program, University of Wyoming, Laramie, WY

Organelles and other intracellular structures must scale with cell size in order to function properly. The functional significance of nuclear size control remains poorly understood, particularly in the context of certain cancers in which the scaling relationship between nuclear and cell size has gone awry. An intriguing possibility is that aberrant nuclear size associated with a disease state might actually be required for cell homeostasis and viability. Elucidating the underlying mechanisms that govern nuclear scaling has proven difficult, due primarily to the lack of experimental approaches capable of precisely manipulating cytoplasmic volume and uncoupling cell size from a developmental or cellular context. To overcome these challenges, we combined microfluidic droplet-generating technology with cell-free *Xenopus* egg extracts. Using this approach, we were able to encapsulate demembrated sperm nuclei in extract droplets and recapitulate nuclear assembly in discrete cytoplasmic volumes. We found that changes in cytoplasmic volume alone, even in the absence of changes in cytoplasmic composition that occur during development and disease progression, could partially explain nuclear scaling as it occurs during development in *Xenopus* embryos. Encapsulated nuclei exhibited asymptotic growth, reaching a volume-dependent steady-state size within two hours post-encapsulation. This is in contrast to

unconfined nuclei, which grew at a constant rate over the same time period. In isovolumetric droplets containing multiple nuclei, nuclear size was dependent upon the total number of encapsulated nuclei. Preliminary results suggest that total nuclear volume is independent of the number of nuclei assembled within a cytoplasmic volume, whereas total nuclear surface area increases with increasing numbers of nuclei. Going forward, this new experimental platform should be of great utility in elucidating the cytoplasmic factors involved in nuclear scaling and will serve as a system to address other important questions of nuclear size regulation.

P1966

Board Number: B479

Over-expression of an export-defective Ltv1 is dominant negative but does not block pre-40S subunit export in *S. cerevisiae*.

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Yeast Ltv1 is a conserved 40S-associated ribosome biogenesis factor that has been proposed to function in small subunit nuclear export. Here we show that Ltv1 has a functional leucine-rich nuclear export signal (NES) at its extreme C-terminus that is both necessary for Crm1 interaction and for Ltv1 export. Over-expression of an Ltv1 lacking its NES (Ltv1 Δ NES) was strongly dominant negative (DN) and resulted in the nuclear accumulation of RpS3-GFP; however, export of the pre-40S was not affected. The dominant negative phenotype of Ltv1 Δ NES over-expression can be suppressed by adding the exogenous PKI NES to Ltv1 Δ NES, showing that Ltv1 must be nuclear to cause the dominant negative phenotype. The DN phenotype could also be suppressed by co-over-expressing RpS3 and its chaperone, Yar1, or by deletion of the RpS3 binding site in Ltv1 Δ NES, suggesting that titration of RpS3 by Ltv1 Δ NES is deleterious in yeast. The dominant negative phenotype did not correlate with a decrease in 40S levels but rather with a reduction in the polysome to monosome ratio, indicating reduced rates of translation. We suggest that titration of RpS3 by excess nuclear Ltv1 interferes with 40S function or with a non-ribosomal function of RpS3.

P1967**Board Number: B480****Simple physical considerations explain the conformational transitions of the FG nucleoporins induced by the nuclear transport factors.**M. Opferman¹, D. Jasnow¹, R. Coalson², A. Zilman^{3,4};¹Physics, Pittsburgh University, Pittsburgh, PA, ²Chemistry, Pittsburgh University, Pittsburgh, PA,³Physics, University of Toronto, Toronto, ON, ⁴Cell and Systems Biology, University of Toronto, Toronto, ON

Nuclear Pore Complex (NPC) is a biological “nano-machine” that controls the macromolecular transport between the cell nucleus and the cytoplasm. It is a remarkable device that combines selectivity with robustness and speed. Unlike many other biological nano-channels, it functions without direct input of metabolic energy and without transitions of the gate from a ‘closed’ to an ‘open’ state during transport. The key aspect of transport is the interaction of the cargo-carrying transport factors with the unfolded, natively unstructured proteins that partially occlude the channel of the NPC and its nuclear and cytoplasmic exits. Mechanistic understanding of the transport through the Nuclear Pore Complex, and in particular its selectivity is still lacking. Conformational transitions of the unfolded proteins of the NPC, induced by the transport factors, have been hypothesized to underlie the transport mechanism and its selectivity. These conformational changes are hard to access in vivo; they have been investigated in vitro generating apparently contradictory results.

We have investigated the biophysical underpinning of these conformational changes, using computational modeling based on the ideas of polymer physics. We show that the differences in the experimentally observed behaviors can be explained by rather general physical factors, such as the attraction strength between the transport factors and the unfolded chains, protein density and the transport factor size. We also show how these general behaviors can be modulated by specific details, such as the aminoacid sequence and the relative arrangement in space of the charged and hydrophobic residues. Finally, we extend the model into a realistic NPC geometry. These results provide new insights into the fundamental principles of transport through the NPC and the control of the behavior of natively unfolded proteins in general.

P1968**Board Number: B481****Establishing a microfluidics platform to watch the deformation of frog oocyte nuclei.**M. Podolski¹, M. Uroz Marimon², M. Kim³, S.K. Tang³, M. Renz⁴;¹Molecular Biophysics and Biochemistry, Yale University, New Haven, CT, ²Department of Biomechanics and Cellular Biophysics, Institute for Bioengineering of Catalonia, Barcelona, Spain, ³Department of Mechanical Engineering, Stanford University, Stanford, CA, ⁴Department of Obstetrics and Gynecology, Albert Einstein College of Medicine, Bronx, NY

Shape and architecture of the mammalian cell nucleus are thought to be maintained by chromatin and nuclear envelope proteins alone. None of the major cytoskeletal proteins, i.e. actin and microtubules, seem to be present in the cell nucleus in considerable amounts. Recently, however, there has been increasing evidence for nuclear actin. This nuclear actin may predominantly exist in its monomeric globular form and may be as such involved in transcription processes. So far, no role for polymerized fibrous actin in the mammalian cell nucleus has been described. It is known, on the other hand, that the actin content in the giant cell nuclei of amphibian oocytes is much higher than in other cell nuclei. The lack of specific export allows the actin to accumulate and consequently polymerize. In frog oocyte nuclei, actin forms a fibrous meshwork morphologically similar to its cytoplasmic counterpart. This actin meshwork may be an inevitable scaffold for the up to 400- μ m measuring frog oocyte nuclei needed for additional mechanical support which cannot be sufficiently provided by chromatin and nuclear envelope proteins alone.

Here, we describe a microfluidics approach to deform frog oocyte nuclei. We provoke cell nucleus deformation by flowing cells through microfluidic channels comprising narrow spaces and orifices. The microfluidic channels used permit high-resolution fluorescence imaging. With these tools, we characterize structural rearrangements of frog oocyte nuclei during and after the imposed deformations.

P1969**Board Number: B482****Cytonuclear transport defect underlies C9ORF72 ALS/FTD neuronal injury in human neurons.**C.J. Donnelly^{1,2}, K. Zhang¹, A.R. Haeusler³, J. Wang³, T.E. Lloyd^{1,4}, R. Sattler^{1,2}, J.D. Rothstein^{1,2,4};¹Neurology, Johns Hopkins University, Baltimore, MD, ²Brain Science Institute, Johns Hopkins University, Baltimore, MD, ³Biochemistry and Molecular Biology, Johns Hopkins University, Baltimore, MD, ⁴Neuroscience, Johns Hopkins University, Baltimore, MD

A hexanucleotide repeat expansion in the C9ORF72 gene has recently been identified as the most common known genetic cause of familial and sporadic amyotrophic lateral sclerosis (ALS) and

frontotemporal dementia (FTD). Recent evidence employing iPS-neurons from C9ORF72 ALS patients suggests that the GGGGCC RNA products transcribed from the repeat expansion form complex G-quartet RNA structures and are neurotoxic. Similar to other repeat expansion disorders (e.g., myotonic dystrophy type I and II), the C9ORF72 GGGGCC_{exp} RNA can sequester nuclear factors, including RNA binding proteins, and we hypothesize that these aberrant interactions are the primary cause of C9ORF72 neurotoxicity. Previous work from our laboratory has indicated that the GGGGCC RNA interacts with RanGAP1, a regulator of Ran-mediated cyto-nuclear trafficking. We have also found that RanGAP1 is a robust suppressor of neurotoxicity in a *Drosophila* model system that overexpresses GGGGCC RNA. Consistent with a RanGAP1 loss-of-function model, both the G4C2 *Drosophila* model and C9ORF72 iPS-neurons show reduced nuclear localization of NLS-containing reporters and that iPS-neurons from C9ORF72 ALS/FTD patients exhibit perturbed Ran protein gradients. The Ran and cytonuclear trafficking deficits observed in C9ORF72 ALS iPS-neurons can be rescued by enhancing neuronal RanGAP1 levels or by treatment with antisense oligonucleotides that target GGGGCC repeat-containing RNAs and small molecules that bind G-quartet RNA structures to prevent any interaction with endogenous proteins. Taken together, these studies strongly support an RNA gain-of-function mechanism underlying C9ORF72 neurodegeneration. Moreover, we show, for the first time, that the toxic GGGGCC_{exp} RNA reduces the nuclear import of classical NLS-containing proteins via an aberrant interaction with RanGAP1. Since cytoplasmic inclusions of predominantly nuclear proteins is the most pronounced pathology in ALS and FTD patient tissue, cytonuclear trafficking deficits might be a common pathway in all ALS/FTD disease pathogenesis.

P1970

Board Number: B483

Nuclear import and DNA damage recognition of Poly(ADP-ribose) Polymerase-2.

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Environmental factors and normal genome replication result in DNA damage that must be repaired to ensure the health of an organism. There are many DNA repair pathways that maintain genome integrity, including; single-strand break repair (SSBR), base excision repair (BER), and homologous recombination (HR)[3,10,11]. An essential set of proteins contributing to DNA damage repair is the poly(ADP-ribose) polymerases or PARPs. The PARP superfamily has 17 members, of which PARPs 1, 2, and 3 are DNA damage-dependent PARPs. Upon the recognition and binding of DNA damage, DNA damage-dependent PARPs increase their catalytic activity several fold generating long, branched polymer of poly(ADP-ribose) (PAR). PAR is a highly negatively charged posttranslational modification and the addition of this polymer onto a target protein can modulate structure, function, and the biochemical properties of the target protein. The current body of PARP literature emphasizes the DNA-dependent detection and activation of PARP-1. However, the essential roles of both PARP-1 and PARP-2 are highlighted by the

embryonic lethality of PARP-1-/-PARP-2-/- double knockout mice. PARP-2 is a multi-domain protein composed of an N-terminal region (NTR), a central WGR domain named for the Trp-Gly-Arg motif, and a C-terminal catalytic domain (CAT). In contrast to the homology in the C-terminal regions, WGR and CAT, the NTR of PARP-2 shares negligible sequence homology with other members of the PARP family. We show that the PARP-2 NTR (i) is intrinsically disordered, (ii) is essential for nuclear localization, (iii) is not sufficient or required for localization to cellular sites of DNA damage, and (iv) is required for in vitro DNA binding and activation on a subset of DNA breaks. We have determined biochemically and biophysically that PARP-2 NTR is unique from other PARPs in its intrinsic disorder. A 1.9Å crystal structure of PARP-2 NLS bound to the nuclear transporter, importin α , demonstrates that PARP-2 contains a bipartite NLS motif required for efficient transport into the nucleus. Through live cell microscopy, the crystal structure is verified by monitoring cellular compartmentalization upon disruption of importin alpha cargo binding residues. Interestingly, it is the C-terminal domains of PARP-2 that bind and localize to sites of cellular DNA damage allowing the functions of nuclear import and DNA binding to be partially separable. However, the NTR remains important for fine tuning regulation of catalytic activity and for SSB recognition. Overall, this study highlights the nonredundant roles of DNA damage PARPs and the distinct role of PARP-2 in the DNA damage response.

P1971

Board Number: B484

Nuclear export of β -Dystroglycan.

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Dystroglycan is an essential component of the Dystrophin-Associated Proteins Complex (DAPC), which maintains the integrity of the sarcolemma and connects the extracellular matrix with the actin cytoskeleton. The DAPC also participates in cell signaling processes. Dystroglycan is composed of α and β subunits, which are associated each other in a non-covalently manner. β -Dystroglycan (β -DG) is a transmembrane protein involved in cell signaling, cell adhesion, and cytoskeleton remodeling. Recently our research group demonstrated that β -DG is transported to the nucleus through a nuclear localization signal (NLS) located in the juxtamembranal region of the protein.

In this study, we present the identification and characterization of a nuclear export signal (NES) in β -DG. In silico analysis identified a putative NES located in the residues 763ILLIAGIIAM772. Treatment of C2C12 cells with leptomycin B, an inhibitor of CRM1 exportin resulted in nuclear accumulation of both endogenous β -DG and GFP- β DG fusion protein, which implies that CRM1 exportin mediates the nuclear export of β -DG. Likewise inactivation of the NES by site-directed mutagenesis caused nuclear accumulation of GFP- β -DG. Finally we demonstrated in vitro interaction of CRM1 exportin with β -DG. Overall, our results suggest that β -DG has a functional NES that is recognized by CRM1 to export the protein from the nucleus to the cytoplasm. The nuclear export of β -DG may be important to modulate its levels in different cell compartments.

Nuclear Bodies and Dynamics

P1972

Board Number: B485

Mobility of mRNA in the nucleus as seen by simultaneous 3D single-molecule real time microscopy (3D-SMRT).

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The ability to image single molecules, such as proteins or RNAs, in the living cell has opened a window for direct visualization of its inner workings. Due to the limited focal depth of conventional light microscopes, however, three-dimensional images must be assembled from a series of images taken along the optical-axis of the microscope over time. The relatively slow acquisition of sequential z-stack images results in a time-difference between the first and subsequent images that limits the accurate 3D recording of rapidly moving single molecules. This can be overcome by simultaneously imaging an entire volume consistent of multiple focal planes.^{1,1a}

Here, we describe simultaneous single RNA-molecule real time (3D-SMRT) microscopy as a way to track the entire distribution of endogenously labeled mRNA within the nucleus of a live mammalian cell. 3D-SMRT microscopy combines the power of multi-focal-plane microscopy with precise registration between fluorescently labeled mRNA, nuclear pore complexes and an intercalating DNA dye that serves as an inherent cellular marker.^{1,2}

By combining redundant geometric local descriptor matching and information entropy based image processing with cell-inherent data we were able to account for aberrations that cannot be accounted for by fiduciary marker-based image registration methods.³ We show that 3D-SMRT microscopy provides exceptional correlation between multiple channels and allows for a quantitative, robust transformation model that globally optimizes image alignment to within half-pixel precision.

P1973**Board Number: B486****New Promising Approach to Karyotype Determination in *Amoeba proteus*.**Y. Podlipaeva¹, S. Demin¹, S. Skarlato¹, A. Yudin¹, M. Berdieva¹, A. Goodkov¹;¹Institute of Cytology, Russian Academy of Sciences, St.Petersburg, Russia

Amoeba proteus-type amebas, the classic model organisms for cell biology studies, are obligatory agamic lower eukaryotes which are characterized by pronounced epigenetic heredity and variability. These unicellular organisms have been under study for about 200 years; however, their chromosome numbers and ploidy level still remain enigmatic. Traditional light and electron microscopical data suggest that the nucleus of *A. proteus* contains about 500 or even more small chromosomes and therefore is likely to be polyploid. Studies of nuclear DNA content during the cell cycle do not shed light on the problem of ploidy because these amebas lack the G₁-phase and the S-phase starts directly after karyokinesis. At the end of the S-phase, the DNA content can increase up to 3.9 times, i.e. the so-called DNA hyper-replication takes place; however, in the G₂-phase DNA is partly eliminated. In mass ameba cultures, one dividing cell can be found per approximately 1000 non-dividing organisms. Thus, the cell cycle synchronization is an important requirement for investigation of the mitotic chromosomes in amebas. In our studies, the *A. proteus* culture, strain B, was synchronized by feeding via pinocytosis on quail egg albumen (Podlipaeva et al., 2013) which allowed: (a) to achieve the high level of cell cycle synchronization; (b) to obtain significant amount of cells at certain mitotic phases, the metaphase in particular; (c) to avoid “background noise” caused by dense food remnants in the digestive vacuoles when amebas were fed with the small ciliates *Tetrahymena*. In our recent experiments, the so-called high-pressure squash technique specifically modified for karyotype studies in amebas allowed to visualize about 23-24 pairs of metaphase chromosomes, which is significantly less than it had been reported for *A. proteus* earlier.

P1974**Board Number: B487****Telomere dysfunction at the nucleotide level: repeat identity in crisis and escape.**. Bennett¹, N. Liu¹, Y. Hu¹, M. Moravec², E. Rodriguez¹, A. Claus², M.C. King¹;¹Department of Cell Biology, Yale University, New Haven, CT, ²Eidgenössische Technische Hochschule Zürich (ETHZ), Institute of Biochemistry (IBC), Otto-Stern-Weg, Switzerland

We have developed a high-throughput sequencing approach that enables us to exactly determine telomeric repeat sequences from tens of thousands of individual *S. pombe* telomeres. To define the molecular changes needed for cells to adopt the alternative lengthening of telomeres (ALT) pathway, we sequenced telomeres from serially cultured cells lacking the telomerase recruitment factor Ccq1. Telomeres from freshly germinated ccq1Δ cells have a highly diverse telomeric sequence content driven by recombination. We find that improved growth, increased telomere length and canonical telomere sequence content in survivors correlates with the derepression of telomeric repeat-containing RNA

(TERRA). Degradation of TERRA in *ccq1Δ* survivors forces a return to a growth crisis. These results suggest that production of TERRA represents a key step in the adoption of the ALT pathway, which has important implications for understanding mechanisms of selection and maintenance of ALT-positive tumor cells.

P1975

Board Number: B488

Dynamic and structural properties of interphase chromatin mapped in vivo with fluorescence correlation spectroscopy and quantitative modelling.

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The three-dimensional organization of chromosomes of eukaryotic interphase cells is emerging as an important parameter for the regulation of storage, replication and expression of the genome. While a range of techniques like electron microscopy (EM), fluorescence in situ hybridization (FISH), or chromosome conformation capture techniques (3C, 4C, 5C, HiC, or the novel targeted chromatin capture, T2C) have been very successfully used to study chromosomal architecture, many details of structural and especially of dynamic properties remain unresolved. Here, we present a novel approach to dissect intramolecular polymer dynamics from fluorescence intensity fluctuations measured with fluorescence correlation spectroscopy (FCS) to investigate the higher order chromatin dynamics in living cells. Using fluorescently tagged linker histone H1 and core histones H2A and H2B as tracer molecules, we find distinct chromatin relaxation times of ~160 ms for open and ~90 ms for dense chromatin areas, corresponding to radii of gyration of 240 and 300 nm for the topologically independent chromatin units. According to their genomic content of ~1 Mb, these units correspond to the distinct topologically associating domains (TADs) found recently by 3C-derived techniques and to subchromosomal domains seen earlier by FISH and in vivo chromatin labelling. We have also obtained light-sheet microscopy-based FCS maps of chromatin domain dynamics. Based on these results, we have developed a quantitative analytical and numerical model of chromatin dynamics that provides access to mass density, persistence length and topological information of chromatin. It allows to extract these parameters from dynamics and 5C/HiC results, to predict chromatin conformation and distance data, and to identify complex looping as crucial for domain formation. Data and model suggest the existence of several connected loops of ~100 kb each per domain. Especially in combination with the recently developed highly selective T2C method (see abstract T.A. Knoch & M. Wachsmuth, "Determination of the three-dimensional organization of chromatin by modelling-supported targeted chromosomal interaction capture (T2C)"), which provides very good signal-to-noise ratio at high genomic resolution, we present a comprehensive systematic approach for the understanding of chromatin dynamics and its relation to structure as well as for insight into the impact on gene regulation.

P1976**Board Number: B489****The Mechanics of Nuclear Shaping.**Y. Li¹, D. Lovett¹, Q. Zhang¹, R. Kuchibhotla¹, S. Neelam², R. Zhu³, G.G. Gundersen³, R.B. Dickinson¹, T.P. Lele¹;¹Chemical Engineering, University of Florida, Gainesville, FL, ²Biomedical Engineering, University of Florida, Gainesville, FL, ³Department of Pathology and Cell Biology, Columbia University, New York, NY

The nuclei in cultured cells are commonly flat, but how the nucleus becomes flattened during cell spreading remains unclear. To answer this question, we imaged NIH 3T3 fibroblasts with a confocal microscope at different stages of spreading and quantified the shape changes of nuclei. Surprisingly, we find that actomyosin forces, microtubule-based forces and intermediate filaments, as well as the LINC complex, are all dispensable for nuclear flattening. Remarkably, nuclear height correlated tightly with the degree of cell spreading. Independent of the type of cytoskeletal force perturbed, the nucleus is flat unless the perturbation prevents initial cell spreading, or rounds up a spread cell. Our computational model shows that F-actin flow due to polymerization from the apical cortex, together with viscous transmission of stress from the moving cell boundary to the nuclear surface, is sufficient to translate and flatten the nucleus, even in the absence of actomyosin contractility.

P1977**Board Number: B490****Novel nuclear localization and function of the actin bundling protein Fascin.**C.M. Groen¹, T.L. Tootle²;¹Anatomy and Cell Biology, Univ Iowa-Carver Coll Med, Iowa City, IA, ²Department of Anatomy and Cell Biology, University of Iowa Carver College of Medicine, Iowa City, IA

The actin regulator Fascin is a critical factor in a number of developmental processes and disease states, such as neurogenesis and cancer. Fascin's roles in these biological processes have largely been attributed to its activity as a cytoplasmic actin bundler, although more recent work suggests that Fascin also has bundling-independent functions. *Drosophila* oogenesis is an ideal system to uncover the function and regulation of cytoskeletal regulators like Fascin (*Drosophila* Singed). Late stage oogenesis requires dynamic actin remodeling events, which include formation of parallel actin filament bundles. Fascin is required for formation of these actin bundles, and loss of Fascin results in female sterility. While Fascin has previously only been shown to function in the cytoplasm, we find that Fascin acts at new cellular sites during *Drosophila* oogenesis. Fascin exhibits weak localization within the nucleus, a previously unreported phenomenon in any system. As development progresses, Fascin more strongly localizes within the nucleus and subsequently undergoes a dramatic re-localization to the nuclear periphery. This dynamic subcellular distribution suggests that Fascin's localization is developmentally regulated. Indeed, we find that loss of lipid signals called prostaglandins (PGs) alters the subcellular localization of Fascin, likely by modifying Fascin's phosphorylation state. When PG signaling is lost,

Fascin initially exhibits a stronger nuclear signal and becomes progressively less associated with the nucleus. In addition, Fascin fails to re-localize to the nuclear periphery at S13 upon loss of PGs. These data suggest that Fascin has important functional roles in the nucleus, and that these novel activities are regulated by PGs. Indeed, we find that disruption of nuclear Fascin affects nucleolar shape and size. When PGs are lost and Fascin is elevated in the nucleus, the nucleolus becomes greatly enlarged and fragmented. By contrast, when Fascin is lost, the nucleolus becomes much more dispersed and unstructured. Importantly, this is not due to loss of cytoplasmic actin bundle formation, as two other *Drosophila* mutants that lack bundles exhibit normal nucleolar structure. Current efforts focus on further elucidating the mechanism by which PGs regulate Fascin and determining the role(s) of Fascin in the nucleus and at the nuclear periphery, including possible associations with nuclear actin. The novel roles of Fascin in the nucleus and at the nuclear periphery are likely contributing to its activities in other tissues and organisms, including during cancer initiation and progression.

P1978

Board Number: B491

Nucleolar assembly and growth are governed by a concentration-dependent phase transition.

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Eukaryotic cells exhibit a remarkable degree of spatial organization. In addition to classical membrane-bound organelles, the cytoplasm and nucleoplasm contain membrane-less, liquid-like droplets of RNA and protein, many of which function in regulating RNA metabolism. How such structures assemble and stably persist without a physical boundary holding them together, and how cells might regulate this process, are still unclear. Here, we use quantitative imaging of live *C. elegans* embryos combined with analytical theory and numerical simulations to investigate the assembly and growth dynamics of the nucleolus, a non-membrane bound organelle important for cell size homeostasis. We find that nucleolar components condense into nucleoli only above a threshold concentration and that the final size of the organelle depends linearly on concentration. Furthermore, the growth kinetics of nucleoli are consistent with a phase separation model in which the thermodynamic attraction between nucleolar components results in stable, coherent droplets. Our results suggest that phase transitions could play a general role in functionally organizing the nucleoplasm/cytoplasm and generating organelles of appropriate size.

P1979**Board Number: B492****Visualization of large scale genomic data in single cells uncovers the functional organization of chromosomes.**T.R. Luperchio¹, X. Wong¹, M. Hallstead², R. Ach³, K. Pekrun³, N. Yamada³, K.L. Reddy¹;¹Biological Chemistry and Center for Epigenetics, Johns Hopkins University School of Medicine, Baltimore, MD, ²UC Davis, Davis, CA, ³Agilent Technologies, Agilent Laboratories, Santa Clara, CA

Nuclear structure and scaffolding have been implicated in expression and regulation of the genome. Discrete domains of chromatin exist within the nuclear volume, and are suggested to be organized by patterns of gene activity. The nuclear periphery, which consists of the inner nuclear membrane and associated proteins, forms a sub-nuclear compartment that is mostly associated with transcriptionally repressed chromatin and low gene expression. Previous studies from our lab and others have shown that repositioning genes to the nuclear periphery is sufficient to induce transcriptional repression; these regions of chromatin that come in molecular contact with the nuclear periphery are called Lamin Associated Domains (LADs). Our current work highlights the relationship of LADs, the epigenome and three-dimensional architecture in development and potential disease phenotypes. LADs are dynamic and serve as a mechanism for regulation of specific gene sets during cellular programs and these dynamics are both seen in populations and single cells. We hypothesized that LAD patterns are reflective of and influenced by higher order chromatin structure and that higher order chromatin structure and epigenetic signatures are closely and functionally linked. Visual studies of LAD architecture in single cells using novel oligonucleotide DNA probes underscores these relationships, and also defines a novel compartment at the nuclear periphery where Lamina Associated Domains are restricted.

P1980**Board Number: B493****Increased age is associated with polyploidy and aneuploidy on neuronal nuclei from mouse brain cortex.**T. Gonçalves do Carmo Oliveira¹, H. Ferreira Rodrigues¹, M. Mondin², T. Andrade de Souza¹, F.G. Ghiraldini³, M. Silveira Mello³, A. da Silva Moraes^{1,4};¹Institute of Biomedical Sciences, Federal University of Uberlandia, Uberlandia, Brazil, ²Department of Genetics, Superior Agriculture School Luiz de Queiroz, University of Sao Paulo, Piracicaba, Brazil, ³Department of Structural and Functional Biology, UNICAMP, Campinas, Brazil, ⁴Federal University of Uberlandia, Uberlandia, Brazil

Background Polyploidy, in mammals, is a phenomenon classically observed in hepatic and muscular cells. However, some previous works show evidence of polyploidy, as well as aneuploidies in brain cells from mouse, rats, and humans. It seems to exist a positive correlation between aging and increased ploidy degrees in some tissues. However, attempts to detect age-related changes in ploidy levels in

neural cells have not been conclusive. **Aims** Look for evidences of polyploidy and aneuploidy on cortical neurons from mice and evaluate if such events are influenced by age. **Methods** Neuronal nuclei were isolated from brain cortices from young and aged mice, followed by flow cytometry of propidium iodide-stained nuclei, image analysis of Feulgen-stained nuclei smeared on histological slides, and telomere analysis after FISH. All experiments involving animal handling and care were approved by the Institution's Committee on Ethics for Animal Experimentation. **Results** Both image and flow cytometry analyses provided evidence of both, aneuploidy and polyploidy in neuronal nuclei, as well as an age-related increase in both phenomena. A higher number of telomeric spots per nucleus in neurons from older mice are in accordance to these data. **Conclusion** Present data show evidence of both polyploidy and aneuploidy, and it is possible that the number of polyploid, as well as aneuploid cells, or the number of individual chromosomes in some cells increases with age. We believe that the increased aneuploidy is linked to impaired maintenance of chromosome structure, and may positively correlate with cognitive decline frequently observed in the elderly. The role of increased ploidy in cells from aged individuals is still an unresolved issue, but may correlate positively with the onset of neurodegenerative disorders like Alzheimer's and Parkinson's diseases.

P1981

Board Number: B494

The Biogenesis of Small Nuclear Ribonucleoproteins in *Arabidopsis Thaliana*.

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The nucleus is the center for all inheritable genetic information. Genetic information regulates everything from form and function to the survival and evolution of life. The nucleus contains a number of distinct non-membranous subcompartments called nuclear bodies. Two examples of such compartments are the nucleolus and Cajal bodies. These subcompartments are formed by a unique set of proteins that carry out specific functions. One such protein is the Survival of Motor Neuron protein (SMN). In animals, mutations in this protein have been implicated to the genetic recessive disorder called Spinal Muscular Atrophy (SMA). SMA is the leading disorder for mortality in children under the age of 2 years. SMN is part of a large protein complex, called the SMN Complex, which is essential for RNA splicing in all eukaryotic cells. SMN has been conserved evolutionary through several organisms ranging from humans to mice, flies, worms and plants. However, little is know about the biology of this pathway in plants. The goal of my project is to study the role of SMN during plant development using *Arabidopsis Thaliana* as a model organism system. Specifically, i) I will use genetic and cellular approaches to characterize the effects of *Arabidopsis* SMN (AtSMN) mutants in the biogenesis of different RNAs. ii) I will analyze, in vivo, the localization of the AtSMN protein at the subcellular level in wild type and in different genetic backgrounds. During the last two semesters I have identified four independent mutations on the AtSMN gene using the Polymerase Chain Reaction method. I have been making genetic crosses using different mutants and nuclear fluorescent markers to study the cell nucleus in my AtSMN mutant plants. Currently, I am cloning the AtSMN gene to rescue SMN mutant

flies to demonstrate that *Arabidopsis* is an excellent model to study the underlying basic biology for SMA.

P1982

Board Number: B495

Characterization of the Spindle Assembly Checkpoint in *S. cerevisiae*.

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The Spindle Assembly Checkpoint (SAC) regulates the metaphase-to-anaphase transition when chromosomes are not properly attached to the mitotic spindle. In *S. cerevisiae*, at the end of G2 phase, the bud is large and the nucleus stretches and divides in two to generate the haploid nuclei of mother and daughter (bud). In this process, the nuclear envelope does not break down. A stable Medial Nuclear Division (MND) intermediate can be imposed by inhibiting activation of the Anaphase-Promoting Complex/Cyclosome (APC/C). We study the effect of SAC using strains in which the APC activator Cdc20 is under the control of a methionine-repressible promoter. Upon arrest, we observe that the nuclear envelope continually extends across the bud neck while chromatin “transits” every 2-10 min between the two domains of the nucleus. During this period (which can last for hours), a short spindle transects the chromatin mass. Moreover, the nucleolus remains attached to the nuclear envelope in the mother cell and the spindle pole bodies repeatedly traverse the bud neck. Especially the latter observation is surprising since the bud neck restricts diffusion of membrane proteins between mother and bud.

P1983

Board Number: B500

3D organization of chromatin in the macronucleus of the ciliate *Bursaria truncatella*.

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Ciliated protists contain two morphologically and functionally different types of nuclei in each cell: a germinal functionally inert micronucleus, and a somatic macronucleus, which is DNA-rich and transcriptionally active during vegetative growth. In contrast to genomes of metazoan cells, the macronuclear genomes are represented by sets of relatively short DNA molecules (minichromosomes) either of “gene size” (0.5-25 kb) or of subchromosomal size (from several tens up to several hundred kb). For this reason, ciliate macronuclei are a convenient object to study the peculiarities of chromatin

and nucleolus morphology in the nuclei with noncanonical genome organization. In particular, non-classical nucleolar organization was observed in *Stylonychia lemnae* (“gene-sized” species) and in *Didinium nasutum* (“subchromosomal” species) macronuclei [1-3].

In this work we studied 3D organization of chromatin in the macronucleus of the ciliate *Bursaria truncatella* using electron microscopy (EM), confocal microscopy, and 3D electron microscopic reconstruction on the basis of serial ultrathin sections [4]. The macronuclear *B. truncatella* DNA molecules vary in size from 50 to about 350 kb. EM data show that in interphase macronucleus these DNAs are packed into chromatin bodies 0.06-0.2 μm in size. During encystment or under unfavourable conditions (e.g. starvation) some chromatin bodies increase in size and aggregate, forming long anastomotic chromatin threads 0.1-0.2 μm thick. 3D models showed that such threads are morphologically similar to the chromoneme fibres observed in the nuclei of higher eukaryotes. These processes only occur in specific regions located axially in the central part of *B. truncatella* macronucleus.

Using BrdU label and confocal microscopy we showed that late replicating genes are mainly located in the central regions of *B. truncatella* macronucleus. On the contrary, early replicating genes are at the periphery of the macronucleus, where no aggregation of chromatin bodies occurs. Our data correlate well with the fact that in metazoan nuclei late- and early-replicating genes are located in the heterochromatin and euchromatin nuclear domains, respectively. However, unlike the metazoan nuclei, early-replicating chromatin is located at the periphery of *B. truncatella* macronucleus, whereas late-replicating chromatin – in its central regions. The data obtained show that the architecture of the nuclei with organization of genome in short minichromosomes can differ from that of metazoan nuclei. RFBR grant 11-04-01967.

Leonova et al., Protist, **157**:391-400, 2006

Leonova et al., Biol. Res., **46**: 69-74, 2013

Postberg et al., Chromosome Res. **14**:161-175, 2006

Leonova et al., Docl. Biochem. Biophys., **391**:208-211, 2003

P1984

Board Number: B501

The DEAH-box helicase Dhr1 Dissociates U3 snoRNA from the pre-rRNA to promote folding of the central pseudoknot.

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A central question in ribosome biogenesis is how RNA-RNA and RNA-protein structural rearrangements are coordinated. In eukaryotes, the highly conserved U3 snoRNA base-pairs with the pre rRNA to

coordinate early cleavage and folding events within pre-40S particles. U3 interactions with the 5' ETS region of the pre-rRNA are essential for cleavage at sites A0, A1 and A2, which generate the 20S, an 18S precursor, and separate the large and small subunit precursors. U3 binds multiple sites in the pre-rRNA, bringing distant elements together. In particular, U3 promotes a long-range interaction termed the central pseudoknot (CPK), a universally conserved architectural feature of the small ribosomal subunit. However, binding of U3 is mutually exclusive with folding of the mature CPK structure, necessitating its removal during pre-40S maturation. Here, we provide genetic and physical evidence that Dhr1 is the helicase responsible for U3 displacement. Active site mutations in Dhr1 trapped a novel ~50S particle containing U3 snoRNA, the core SSU Processome factors Mpp10 and Imp4 and 20S and 21S pre-rRNA. These RNAs have undergone U3-dependent cleavage at sites A0 and A1 but are only partially cleaved at site A2. Chemical probing was consistent with U3 snoRNA remaining base-paired with pre-rRNA. UV crosslinking identified Dhr1 binding sites in the 5' region of U3 snoRNA and U29, C39, G47 and A48 as nucleotides that were directly crosslinked to Dhr1. These residues are adjacent to U3 box A, which is believed to bind across the CPK, and to U3 5'-hinge, a sequence that base-pairs to the 5' ETS. Separately, we identified mutations in U3 snoRNA that suppressed the growth defect of a *dhr1* cold-sensitive mutant. The suppressing mutations in U3 mapped to residues 1 through 28, immediately upstream of the Dhr1 binding site. Finally, we show that purified Dhr1 is an active RNA helicase that can displace a model U3 snoRNA-pre-rRNA duplex. Together, these data strongly suggest that Dhr1 promotes the release of U3 snoRNA to promote the formation of the CPK and separation of pre-40S from the early pre-ribosome.

P1985

Board Number: B502

Reproduction of FC/DFC units in nucleoli.

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Nucleoli are formed on the basis of Nucleolus Organizer Regions (NORs), i.e., the clusters of ribosomal genes coding for the ribosomal particles. FC/DFC units are key structural components of the nucleoli, where rDNA is transcribed, and the transcripts are processed. In the present study we followed cell cycle related changes of these units in vivo using a human sarcoma derived cell line with stable expression of GFP-RPA43 (a subunit of RNA polymerase I, pol I) and RFP-PCNA (the sliding clamp protein). Employing CLEM, we could identify the FC/DFC units previously observed in the live cells also on the thin plastic sections. According to our data, in early S phase, when transcriptionally active ribosomal genes are replicated, the units occasionally divided or lost pol I. Remarkably, although the units multiplied from early to late S phase, their number in that period increased only by about 70%. After the cell division, the units gradually unfolded from the NORs, and around the middle of G1 phase their number in each of

the daughter cells exceeded one half of the number observed in the maternal cell in G2. The peculiar mode of reproduction of FC/DFC units suggests that a considerable subset of ribosomal genes remain transcriptionally silent from late S phase to mitosis, but get activated in the postmitotic daughter cells.

P1986

Board Number: B503

Replication and transcription machineries are efficiently separated in nucleoli.

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In mammalian cells, ribosomal genes coding for the 18S, 5.8S and 28S rRNAs are transcribed within the nucleolar structures called FC/DFC units (i.e. Fibrillar Centers with adjacent Dense Fibrillar Components). Since these genes are intensively transcribed throughout interphase, some kind of mutual adjustment is needed to avoid collision of the DNA polymerase and RNA polymerase machineries. In this work, we measured correlation of various replication and transcription signals in the nucleoli of HeLa, HT-1080 and NIH 3T3 cells employing a specially devised software for analysis of confocal images. Using a stable cell line expressing GFP-RPA43 (subunit of RNA polymerase I) and RFP-PCNA (the sliding clamp protein) based on HT-1080 cells, we found that replication and transcription signals are more efficiently separated in nucleoli than in the nucleoplasm. Analysis of single molecule localization microscopy (SMLM) images indicated that transcriptionally active FC/DFC units did not incorporate DNA nucleotides. Moreover, comparing distribution of distances between the transcription (FU) and replication (EdU) signals on the one side and between fibrillarin (which is a structural component of DFC) and EdU signals on the other, shows that EdU signals tends to localize closer to fibrillarin than FU signals. Our data indicate that FC/DFC units may provide a structural basis for the efficient separation of replication and transcription in the nucleoli. Acknowledgements: The work was supported by the grants: P302/12/1885, 12/G157 and 13-12317J from the Czech Grant Foundation, and UNCE 204022 and Prvoux /1LF1/1 from the Charles University in Prague.

P1987

Board Number: B504

Physical Characterization of a Model Phase-Separated Protein Droplet.

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Non-membrane bound intracellular organelles are associated with a wide range of biological functions. Several of these structures have been shown to be enriched with multi-valent molecules and exhibit liquid-like properties that may represent droplets of phase-separated RNA and/or proteins. Little is

known about the molecular interactions driving the assembly, properties, and function of these organelles. Here, we address this question using a model multi-valent protein system consisting of repeats of the Small Ubiquitin-like Modifier (SUMO) protein and a SUMO-interacting motif (SIM). Solutions of these proteins are found to undergo phase separation into liquid-like droplets, both in vitro and within cytoplasmic extracts. We combine microrheology and quantitative microscopy approaches to determine the viscosity, density and surface tension of these droplets. We also utilize fluorescence recovery after photobleaching (FRAP), fluorescence correlation spectroscopy (FCS) and Dextran partitioning experiments to probe the internal structure and molecular dynamics within these droplets. Our results shed light on how the strength of inter-molecular interactions manifests in mesoscale droplet properties, and lay the groundwork for a comprehensive biophysical picture of intracellular RNA/protein organelles.

P1988

Board Number: B505

Spatial relationship between Bdnf alleles and their parent chromosome territories in neurons upon seizures.

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In the interphase nuclei chromatin is organized in the form of chromosome territories. Moreover, it is known that particular genes are non-randomly located in the nuclear space. From the experiments concerning several types of cells, it is known that the position of gene loci in relation to the parent chromosome territory is not stable, and may change upon various conditions (e.g. during differentiation). However, little is known about this phenomenon in differentiated cells such as neurons. Previously, in the rat model of epilepsy, we showed that the localization of Bdnf gene is non-random in hippocampal neurons. In the control neurons, Bdnf alleles were localized at the nuclear periphery in as many as 50,5% of the nuclei, whereas in the nuclei of the stimulated neurons only 27,9% had the Bdnf alleles at the nuclear periphery. To examine the spatial positioning of the alleles in relation to chromosome 3 territory we performed fluorescent in situ hybridization with the chromosome painting probe and the gene-specific probe. The allele was considered located outside the chromosome territory if the distance from its center to the surface of the territory was greater than 350 nm (the microscope resolution averaged in three dimensions). We observed that in the control animals there were 21,74±0,02% of the nuclei with both Bdnf alleles located outside of its parent chromosome territories, 46,52±0,01% of the nuclei with one of the allele located outside its parent chromosome territory and as many as 31,74±0,02% of the nuclei with none of the alleles located outside of its parent chromosome territory. Such an analysis was also performed for the neurons after seizures. Here we observed that 26,95±0,12% of the nuclei had both of the Bdnf alleles located outside of parent chromosome territory whereas 41,84±0,09% of the nuclei had one of the allele located outside of the chromosome territory. We did not observed any difference as far as the nuclei with none of the alleles located outside of the

territory is concerned. The slight but significant difference in the percentage of the nuclei with the alleles outside the chromosome territory suggests that the alleles tend to loop out from their parent territory upon neuronal stimulation. Presented study was supported by the 'Preludium' grant for young investigators financed by Polish National Science Center (grant number 2011/03/N/NZ3/04516).

Vesicle Docking and Fusion

P1989

Board Number: B507

Rab3/rabphilin is present on a subset of vesicles predestined to dock to RIM clusters at the plasma membrane.

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Insulin is released by regulated exocytosis, which requires secretory vesicles to be docked at the plasma membrane. The number of release ready vesicles at the plasma membrane is therefore rate limiting for hormone secretion. Stable docking is preceded by a loosely tethered state, and we showed recently that this transition occurs within seconds after arrival of the vesicle by recruitment of syntaxin and munc18 to the docking site. The molecular nature of the tethered state is not known and it remains elusive whether the vesicles tether to a preexisting receptor complex in the plasma membrane or attach to random sites. To answer this we quantified GTP-binding rab proteins and their effectors at the docking site by imaging GFP-tagged proteins using TIRF microscopy. Clusters of the rab3 interacting protein RIM and rabphilin existed at docking sites prior to vesicle tethering and docking. A further increase in RIM fluorescence was seen at vesicles during their maturation into the releasable pool, confirming a role of RIM in priming. Vesicles that successfully docked carried rab3 and rabphilin whereas those that only temporarily tethered did not. In contrast, rab27 and its effector granuphilin were present on both types of vesicles. These results suggest that rab3 and rabphilin act on the incoming granules as signals to initiate the docking process. Since RIM is thus far the only protein found to be enriched at the docking site it may act as a docking receptor for the incoming vesicle.

P1990

Board Number: B508

Dynamic modulation of cortical actin at the immunological synapse controls cytotoxic granule secretion.

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Cytotoxic T lymphocytes (CTLs) are an integral part of the immune system as they are responsible for destroying virally infected and tumorigenic cells throughout the body. CTL undergo a dramatic reorganization of the actin and microtubule cytoskeletons upon target recognition, facilitating the polarized secretion of a potent cocktail of lytic proteins that induce target apoptosis. These cytolytic proteins are housed in specialized lysosome-related organelles called “lytic granules.” It is known that CTL direct lytic granule trafficking specifically in the direction of the target by polarizing the centrosome to the CTL/target interface. However, how CTL regulate secretion of lytic granules is still unclear. The actin cortical cytoskeleton plays a role in regulating exocytosis in a number of professional secretory cells. It can act as a barrier-- preventing secretory granules from reaching the plasma membrane. We previously showed that actin clears away from the plasma membrane at the interface between the T cell and the target. However, controversy remains as to the role of actin in secretion at the synapse. Using newly developed Lattice Light Sheet Microscopy, we show that actin cortical density is reduced at the synapse within 1 minute after target recognition. We also find that lytic granules cluster at the centrosome prior to its delivery to the synapse. We hypothesize that the rapid formation and maintenance of an area of reduced cortical actin density at the synapse after target recognition could provide a platform to mediate immediate and facile secretion of lytic granules which are delivered to the synapse in conjunction with the centrosome. Supporting this hypothesis, we also note that CTL manipulate the actin cortical cytoskeleton to prevent secretion. Using TIRF microscopy, we show that following secretion of a few granules, actin cortical density dramatically increases at the synapse. Surprisingly, actin cortical density remains reduced at the synapse lacking proteins that are required to secrete lytic granules, indicating that this actin “wall” forms only after lytic granule secretion. Depolymerization of the actin “wall” with low doses of Latrunculin A results in immediate lytic granule secretion. This suggests that this actin wall could be serving as a barrier to prevent sustained lytic granule secretion toward an individual target cell. CTL are serial killers. In order for this to be true, CTL must regulate the number of granules that are secreted in each target encounter. This way, they maintain a complement of granules to be used against subsequent target cells.

P1991

Board Number: B509

Investigating the regulation of pulmonary surfactant secretion using fluorescence microscopy.

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Alveolar type II (ATII) cells are found in the lining of alveolar air sacs in the mammalian lung. One function of these cells is the secretion of pulmonary surfactant, which is a mixture of lipids and protein that reduces surface tension in the lung and helps to maintain functional air exchange. In cells pulmonary surfactant is stored within large vesicles known as lamellar bodies (LBs), and the calcium-

dependent fusion of these vesicles with the plasma membrane is accomplished using the machinery of regulated exocytosis including SNARE proteins and a host of effectors and regulatory partners. We are developing a model system for studying surfactant secretion in A549 cells, a human lung carcinoma line derived from ATII cells and that shares many features with ATII cells. Our approach is to visualize LB fusion and the relative localization of associated proteins in live cells using total internal reflection fluorescence microscopy. Here we focus on two families of calcium-sensitive membrane-binding proteins that are potential regulators of LB fusion: the synaptotagmins and annexins. We find that A549 cells exhibit LB fusion after stimulation using hypo-osmotic shock, which induces cell swelling and membrane stretching, consistent with ATII cell physiological response to breathing and lung expansion. We have not observed evidence that the synaptotagmin isoforms expressed in lung tissue colocalize with sites of LB fusion, suggesting that this protein family may not play a local role in the regulation of LB fusion. However, annexin A7 appears to localize to sites of LB exocytosis concomitant with membrane fusion. Annexin A7 does not appear on the membrane prior to fusion, at our time resolution, and this suggests annexin may not initiate fusion but is involved in a later phase. Interestingly, we observe actin recruitment to sites of LB fusion, and annexins have previously been suggested to mediate actin polymerization at lipid membranes. We are currently investigating this possible relationship and other functions of the annexins in regulated pulmonary surfactant secretion.

P1992

Board Number: B510

Clustering of L-type Ca²⁺-channels promotes exocytosis of individual secretory granules.

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Aim:Ca²⁺ entry through voltage gated L-type calcium channels triggers release of insulin-containing secretory granules. Electrophysiological data suggest that channel clustering near docked granules is critical for exocytosis and physiological insulin secretion. A certain number of clustering is thought to be mediated by binding of the α 1 subunit of L-type channels to SNARE proteins through its synaptic protein interaction site (synprint). However, direct evidence for channel clustering near granules is lacking.

Materials and methods:Distribution of the EGFP-labeled Ca²⁺-channel subunit Cav1.2 was imaged together with a granule marker in rat insulin secreting Ins1 cells using TIRF-microscopy. Localized Ca²⁺ influx was imaged using the calcium sensor Ca²⁺ sensor (lyn-GECO-R, membrane-targeted) or Fluo5F in EGTA loaded cells (Ins1, mouse and human pancreatic beta cells). Ca²⁺-currents were quantified using patch clamp and local Ca²⁺-gradients estimated using a deterministic model.**Results:**Cav1.2-EGFP formed clusters on a hazy background that partially colocalized with docked granules. Image analysis indicated specific binding of Cav1.2-EGFP to granules, which was confirmed by Single molecule imaging that

demonstrated reduced mobility of Cav1.2-EGFP near docked granules. This binding was not detectable when the synprint fragment was coexpressed, suggesting that the peptide competes with and uncouples Cav1.2 from the exocytosis machinery. Ca^{2+} -channel clusters formed at granules at least 60 s after their arrival at the plasma membrane, and rapidly dispersed after exocytosis. In depolarized cells, docked granules that exocytosed (responders) carried significantly more Cav1.2-EGFP than those that failed to do so (failures). Visualization of Ca^{2+} -influx indicated localized Ca^{2+} -entry with faster $[\text{Ca}^{2+}]$ increase at responder granules compared with failures or random sites. Modeling of Ca^{2+} -influx confirmed that the observed rates of Ca^{2+} -entry and distribution are consistent with localized Ca^{2+} -influx near granules. The copy number of calcium channels associated with the granule site was estimated by combined fluorescence and current recordings. Common signaling pathways in the pancreatic β -cell do not affect channel clustering. **Conclusions:** In summary, we demonstrate clustering of Cav1.2 channels near a subset of docked insulin granules. This arrangement results in localized calcium entry and selective exocytosis of the associated granules. Channel clustering depends on short term binding via the synprint domain and disrupting this interaction leads to dispersal of the channels away from granules.

P1993

Board Number: B511

Spatial control of H,K-ATPase-containing membrane trafficking and docking by phosphorylation-coupled ezrin-syntaxin 3 interaction.

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The digestive function of the stomach depends on acidification of the gastric lumen. Acid secretion into the lumen is triggered by activation of a cAMP-dependent protein kinase (PKA) cascade, which ultimately results in the insertion of gastric H,K-ATPases into the apical plasma membranes of parietal cells. A coupling protein is ezrin whose phosphorylation at Ser66 by PKA is required for parietal cell activation (J. Biol. Chem. 2003. 278, 35651-9). However, little is known regarding the molecular mechanism(s) by which ezrin operates in gastric acid secretion. Here we show that phosphorylation of Ser66 induces a conformational change of ezrin which enables its association with syntaxin 3 and provides a spatial cue for H,K-ATPase trafficking. This conformation-dependent association is specific for syntaxin 3 and binding interface and is mapped to the N-terminal region. Biochemical analyses show that inhibition of ezrin phosphorylation at Ser66 prevents ezrin-syntaxin 3 association and insertion of H,K-ATPase into the apical plasma membrane of parietal cells. Using atomic force microscopic analyses, our study revealed that phosphorylation of Ser66 induces unfolding of the ezrin molecule to allow syntaxin 3 binding to its N-terminus. Given the essential role of Stx3 in polarized secretion, our study presents the first evidence in which phosphorylation-induced conformational rearrangement of ezrin provides a spatial cue for polarized membrane trafficking in epithelial cells.

P1994**Board Number: B512****TRIM72/MG53 directly modulates phosphatidylinositol-4,5-bisphosphate 3-kinase signaling to mediate vesicular trafficking during endocytosis and exocytosis.**S. Bhattacharya¹, J. Alloush¹, E. Beck¹, L. Gushchina¹, N.L. Weisleder¹;¹Davis Heart and Lung Research Institute, Ohio State University Medical Center, Columbus, OH

Tripartite motif (TRIM) proteins constitute a family of proteins that all contain a canonical RING finger, B-box and coiled-coil domains which participate in several cellular processes. TRIM72, also known as mitsugumin 53 (MG53), is an essential component of the membrane repair machinery in striated muscles. The TRIM72/MG53 knockout mouse displays compromised muscle membrane repair and *trim72*^{-/-} mice develop myopathy. Due to its essential role in the membrane repair process in striated muscle, TRIM72/MG53 has recently been considered as a therapeutic candidate for the treatment of muscular dystrophy. Following membrane disruption, TRIM72/MG53 translocates to the site of injury to allow resealing of the damaged area. The precise molecular mechanism by which TRIM72/MG53 translocate to the injury site and reseal the damage membrane is clearly not known. Previous studies established that membrane damage increases vesicular trafficking to the site of membrane injury, which is followed by vesicular fusion to patch the damaged portion of the membrane. Previous studies indicate that both endocytosis and exocytosis are important in the membrane repair process, and also that TRIM72/MG53 expression can appear in vesicles that are released from myocytes. In this study we examined the role of TRIM72/MG53 in vesicular endocytosis and exocytosis in C2C12 myoblasts overexpressing TRIM72/MG53. The extent of vesicular endocytosis and exocytosis was measured as internalization of fluorescently tagged dextran and internalization/recycling of fluorescent transferrin using laser scanning confocal microscopy and fluorescence-activated cell sorting (FACS), respectively. Overexpression of TRIM72/MG53 in C2C12 myoblasts increased the total amount of endocytosis of labeled dextran into the myoblasts. Exocytosis as assayed by transferrin internalization and recycling was also increased by expression of TRIM72/MG53. Our recent findings show that TRIM72/MG53 overexpression is sufficient to activate the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) signaling pathway. Therefore, we hypothesized that this TRIM72/MG53 mediated vesicular translocation may be regulated by a PI3K-dependent pathway. To test this hypothesis, dextran uptake and transferrin internalization/recycling were measured in the presence of wortmannin. Wortmannin significantly attenuated dextran uptake and transferrin internalization/recycling in C2C12 myoblasts. To control for any off-target effects of chemical inhibitors, molecular approaches were used to introduce constitutively active PI3K and protein kinase B (AKT) constructs which increased dextran and transferrin trafficking in C2C12 myoblasts similar to TRIM72/MG53. Dominant negative PI3K and AKT constructs prevented TRIM72/MG53 mediated increases in endocytosis and exocytosis. We conclude that TRIM72/MG53 is an important regulator of the PI3K/AKT signaling pathway and positively regulates translocation of vesicles to the site of membrane injury.

P1995**Board Number: B513****The exocyst subunit Sec6 has a role downstream of SNARE assembly.**M.L. Dubuke¹, S. Maniatis², S. Shaffer², M. Munson¹;¹Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA, ²Proteomics and Mass Spectrometry Facility, University of Massachusetts Medical School, Worcester, MA

In eukaryotic cells, membrane-bound vesicles carry protein and lipid cargo between intracellular compartments, to and from the cell surface, and to the extracellular environment. A major unanswered question is how vesicle fusion is tightly controlled to prevent premature or inappropriately localized membrane fusion. Two of the conserved families of proteins required for vesicle fusion at the proper target membrane site are the oligomeric tethering complexes and the SNARE proteins. The exocyst is the exocytic tethering complex that regulates polarized exocytosis, and is proposed to tether vesicles to their sites of fusion. Subsequently, the SNARE proteins mediate membrane fusion. Because the exocytic SNARE proteins show little specificity for their binding partners and are not always localized to sites of membrane fusion, SNARE complex assembly must be highly regulated. Previous work from our laboratory demonstrated that, in vitro, the *Saccharomyces cerevisiae* exocyst subunit Sec6 directly binds the plasma membrane SNARE Sec9 and inhibits the assembly of the binary SNARE complex containing Sec9 and the plasma membrane SNARE Sso1. Sec6 and Sec9 also interact in vivo; therefore, we hypothesize that the interaction between Sec6 and Sec9 regulates the assembly of SNARE complexes at sites of exocytosis upon vesicle arrival. In order to elucidate the role of the Sec6-Sec9 interaction in SNARE complex assembly, we used the zero-length crosslinker EDC (1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride) and mass spectrometry analyses to identify the residues required for binding. We monitored the cross-linking reaction over a range of times from 15 to 120 minutes, and identified a patch of residues on Sec9 between the two SNARE helices; these residues were mutated for further analysis. A re-interpretation of our lab's previous data regarding Sec6's inhibition of Sec9-Sso1 complex formation, combined with new data using the Sec9 mutant protein, suggests that Sec6 is capable of binding to both the binary Sec9-Sso1 SNARE complex and the ternary Sec9-Sso1-Snc2 SNARE complex. The mutations in the Sec9 linker are not sufficient to disrupt the Sec6-Sec9 interaction, but are sufficient to disrupt binding of Sec6 to the binary SNARE complex. These results suggest a new role for Sec6 downstream of SNARE assembly.

P1996**Board Number: B514****The Q-SNARE SNAP-47 regulates trafficking of selected VAMPs.**

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SNAREs constitute the core machinery for membrane fusion. Vesicular SNAREs localize to different intracellular compartments via largely unknown mechanisms. Here we sought to define the regulatory network of TI-VAMP/VAMP7 by a proteomics approach. We were able to confirm previously described interactions and further identified an interaction between VAMP7 and SNAP-47. We found that SNAP-47 preferentially interacts with VAMPs 4, 7 and 8, and is mainly localized to the endoplasmic reticulum, and ERGIC. Yeast two-hybrid screens pulled out syntaxins with SNAP-23 and -29 but not SNAP-47 as baits which instead interacted with proteins involved in budding and transport. Nevertheless, SNAP-47 was coimmunoprecipitated by syntaxin 5 but also syntaxin 1 when this latter is retained in the ER in the absence of Munc18a. A C-terminally deleted form of the protein was impaired in VAMPs interaction and affected the subcellular distribution of VAMP4 and VAMP7. Silencing of SNAP-47 expression further decreased the localization of VAMP4 in the Golgi apparatus. Overexpression of SNAP-47 inhibited the exocytic activity of pHluorin-tagged VAMP7. We conclude that SNAP-47 plays a role in budding and transport through the early secretion pathway of a subset of v-SNAREs to regulate their post-Golgi functional availability.

P1997**Board Number: B515****The golgin coiled-coil proteins tether vesicles.**

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A key step in membrane traffic is tethering, which provides initial association between transport vesicles and their target membranes prior to SNARE-mediated membrane fusion. The golgins are one class of putative vesicle tethering factors found on the Golgi apparatus. Their precise functions have been unclear, partially due to redundancy suggested by mild phenotypes of mutant models. In this work, we adopted a relocation strategy to test for sufficiency rather than necessity. Ten golgin proteins were systematically relocated to the mitochondria through substituting their C-termini, which have been shown to confer Golgi localisation, by a mitochondrial transmembrane domain. We found that individual golgins are capable of capturing ER-derived cargos, Golgi residents, or endosome-to-TGN cargos to the mitochondria. Furthermore, ultrastructural studies show striking accumulation of vesicles around mitochondria decorated with particular golgins. Our results demonstrate for the first time in vivo that

golgins are indeed vesicle tethers, and that they exhibit specificity towards different classes of transport vesicles that travel to or through the Golgi.

P1998

Board Number: B516

The HOPS/Class C Vps complex tethers membranes by binding to a Rab GTPase in one membrane and directly via a curvature-sensing motif to a second, highly-curved membrane.

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Membrane tethering factors are thought to function by simultaneously binding to two apposed membranes. However, the sites on tethered membranes that are bound by tethering factors in order to form membrane-bridging physical linkages remain unknown. Here, we use liposomes and purified proteins to identify the physical interactions needed for membrane tethering by the HOPS/Class C Vps complex, an effector for the yeast vacuolar Rab GTPase Ypt7p. We find that HOPS can tether two low-curvature membranes using its two Ypt7p binding sites – one in its Vps41p subunit, the other in its Vps39p subunit – to bind a molecule of Ypt7p in each of the apposed membranes. HOPS also can tether a low-curvature membrane to a high-curvature membrane by binding to the low-curvature membrane via Ypt7p and to the highly-curved membrane using a curvature-sensing ALPS motif in Vps41p. A protein construct containing only the Ypt7p-binding site and ALPS motif from Vps41p was unable to tether membranes, while a HOPS complex lacking Vps39p was similarly unable to perform tethering. We therefore conclude that HOPS tethers low-curvature membranes to high-curvature membranes by binding via Vps39p to Ypt7p in the low-curvature membrane, and via its ALPS motif to the high-curvature membrane. These results suggest a model for how HOPS tethers both high-curvature membranes (e.g. small transport vesicles) and low-curvature membranes (e.g. late endosomes/multivesicular bodies) at the large, low-curvature vacuole/lysosome.

P1999

Board Number: B517

Conformational flexibility of GCC185 is required for vesicle tethering at the trans Golgi.

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Membrane trafficking involves the collection of cargo molecules into transport vesicles, movement of vesicles along cytoskeletal tracks, and tethering, docking and fusion of vesicles at a target membrane.

Transport vesicle tethering is perhaps, the least understood process in membrane traffic. GCC185 is a trans Golgi-anchored, coiled coil tethering protein required for maintenance of Golgi structure and the receipt of transport vesicles inbound from late endosomes. GCC185 is predicted to be a highly extended protein, and like most other coiled coil tethers, it contains regions of predicted flexibility that may function as a hinge to bring vesicles into close proximity with target membranes. We have used atomic force microscopy (AFM) to analyze the structure of purified human GCC185 protein. The full-length protein (mean length: ~130 nm) as well as its fragments, appear shorter than expected according to structure prediction algorithms. A clear splaying of the dimeric coiled coil is seen near the middle of GCC185, a feature that can serve as a flexible, central joint within this tethering factor. Proximity ligation experiments revealed that GCC185's N- and C-termini can be detected in close proximity (<40 nm) on the surface of the Golgi in cultured cells; proximity was significantly decreased for deletion mutants lacking the splayed, central region. Using purified GCC185 fragments, we detected direct interaction between the N-terminal 358 residues and a C-terminal domain, consistent with the proximity of these regions, detected in cells. Functional rescue of mannose 6-phosphate receptor trafficking in GCC185-depleted cells required the presence of GCC185's central flexible region; replacement of these sequences with unstructured Gly-Gly-Ser repeats yielded the same, complete rescue of mannose 6-phosphate receptor trafficking seen with wildtype GCC185 protein. These experiments provide a detailed, structural analysis of a trans Golgi-associated coiled coil tether, and demonstrate, for the first time, a requirement for central flexibility in transport vesicle tethering at the trans Golgi.

P2000

Board Number: B518

The syntaxin 31-induced gene, LESION SIMULATING DISEASE1 (LSD1), functions in Glycine max defense to the root parasite *Heterodera glycines*.

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The membrane fusion genes alpha soluble NSF attachment protein (α-SNAP) and syntaxin 31 (Gm-SYP38) contribute to Glycine max defense to the plant parasitic nematode *Heterodera glycines*. Accompanying their expression is the transcriptional activation of the defense genes ENHANCED DISEASE SUSCEPTIBILITY1 (EDS1) and NONEXPRESSOR OF PR1 (NPR1) that function in salicylic acid (SA) signaling. These results implicate the added involvement of the antiapoptotic gene LESION SIMULATING DISEASE1 (LSD1). Roots engineered to overexpress Gm-α-SNAP, SYP38, EDS1, NPR1, BOTRYTIS INDUCED KINASE1 (BIK1) and xyloglucan endotransglycosylase/hydrolase (XTH) in the susceptible genotype G. max[Williams 82/PI 518671] have induced Gm-LSD1 (Gm-LSD1-2) transcriptional activity. In reciprocal experiments, roots engineered to overexpress Gm-LSD1-2 in the susceptible genotype G. max[Williams 82/PI 518671] have induced levels of SYP38, EDS1, NPR1, BIK1 and XTH, but not α-SNAP prior to infection. In tests examining the role of Gm-LSD1-2 in defense, its overexpression results in reduced

nematode parasitism. In contrast, RNA interference (RNAi) of Gm-LSD1-2 in the resistant genotype G. max[Peking/PI 548402] results in an 3.24-10.42 fold increase in parasitism. The results identify that Gm-LSD1-2 functions in the defense response. It is proposed that LSD1, as an antiapoptotic protein, may establish an environment whereby the protected, living plant cell could secrete materials in the vicinity of the parasitizing nematode to disarm it. After the targeted incapacitation of the nematode the parasitized cell succumbs to its targeted demise as the infected root region is becoming fortified. The results indicate a similar framework may be in place for other agriculturally important root parasitic nematodes.

P2001

Board Number: B519

Cytoskeleton organizes exocytosis “hotspots” in endocrine cells revealed by spatiotemporal analysis of discrete vesicle fusion events.

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In secretory cells, depolarization induces rapid fusion of secretory vesicles with the plasma membrane, thus releasing neurotransmitters and hormones to mediate important physiological processes (1). Using fluorescent imaging and electrophysiological methods, people have studied the molecular mechanisms underlying vesicle exocytosis. Cytoskeletons have been proposed to regulate transport of vesicles to the plasma membrane (2). The spatial information of the fusion events within a single cell has seldom been systematically investigated due to the lack of a fully automated program to analyze large image datasets. Therefore, how cytoskeletons regulate the spatial profile of fusion sites remains unknown. Here, we performed spatiotemporal analysis of exocytic events recorded from insulin-secreting INS-1 cells under a total internal reflection fluorescence microscope (TIRFM). We statistically prove that individual vesicle fusion events are clustered at “hotspots”. This spatial preference disappears upon pharmacological disruption of either the actin or the microtubular network, which also severely inhibit evoked exocytosis. During long time recording of single cell exocytosis under high glucose stimulation, we observed the changes of exocytosis between different “hot regions”. The results indicate that the biphasic insulin secretion involves the changing of spatial preference of exocytosis over time. By demonstrating that newcomer vesicles are delivered from deep in the cell to the surface membrane and contribute a large proportion of exocytosis, we highlight an unappreciated mechanism, in which the cytoskeleton-dependent transportation of newcomer secretory vesicles instead of the active-zone-like accumulation of pre-docked secretory vesicles organizes exocytosis hotspots in endocrine cells.

Sudhof, T. C., and J. Rizo. 2011. Synaptic vesicle exocytosis. Cold Spring Harbor perspectives in biology 3.

Cingolani, L. A., and Y. Goda. 2008. Actin in action: the interplay between the actin cytoskeleton and synaptic efficacy. Nature reviews. Neuroscience 9:344-356.

P2002**Board Number: B520****Integral membrane protein sorting and degradation is intrinsic to vacuole fusion.**E. McNally¹, C. Brett¹, J. Richard¹;¹Biology, Concordia University, Montreal, QC

Integral Membrane Proteins (IMPs), such as surface receptors and transporters, are essential for signaling and substrate transport across membranes. Consequently, the regulation of IMP expression levels underlies numerous fundamental cellular processes. Therefore, determining how these proteins are degraded is important for a comprehensive understanding of cell physiology. Eukaryotic cells degrade surface IMPs via endocytosis and the Multi Vesicular Body (MVB) pathway, which solves the topological challenge of exposing IMPs to luminal acid hydrolases within the vacuole (or lysosome) for degradation. However, this process does not account for turnover of all IMPs within the cell. For example, it is not understood how IMPs that avoid the MVB machinery, such as vacuole transporters, are degraded. Based on detailed studies of homotypic vacuole fusion, it is known that a portion of the organelle membrane, called the contact zone, is internalized and degraded as a product of the reaction. Thus, we hypothesize that if vacuole IMPs are sorted into the contact zone, then they will be internalized and degraded as a consequence of homotypic membrane fusion. To test this hypothesis in *Saccharomyces cerevisiae*, we monitored transporters during the homotypic vacuole fusion reaction both in vivo and in vitro using time-lapse fluorescence microscopy, a pHulorin-based reporter assay and by Western blotting for products of degradation. We find that most GFP-tagged vacuole transporters are excluded from the contact zone and spared. However, some GFP-tagged transporters tested are actively sorted into the contact zone, internalized and degraded during homotypic vacuole fusion. Importantly, degradation of these IMPs is not affected by mutations that impair the MVB pathway. Ongoing experiments are designed to uncover the mechanisms responsible for labeling and sorting of vacuole IMPs destined for degradation. However, our initial findings confirm the existence of a second, independent cellular pathway responsible for the degradation of intracellular IMPs. Analogous to the role endocytosis in controlling surface IMP levels, this process likely controls the IMP profile that defines organelle identity and function required for normal cellular physiology.

Post-Golgi Trafficking

P2003

Board Number: B521

OVERLAPPING FUNCTIONS OF PI4KII AND PI4KIII β (Fwd) IN DROSOPHILA CELLS.

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Phosphatidylinositol 4-kinases (PI4Ks) synthesize a pool of phosphatidylinositol 4-phosphate (PI4P) that binds and recruits factors required for post-Golgi trafficking. *Drosophila melanogaster* has a single type II enzyme (PI4KII) and two type III enzymes (PI4KIII α and PI4KIII β (Fwd)). Previous studies in our lab have shown that PI4KII and PI4KIII β (fwd) are non-essential since their null mutants are viable. Fwd is localized to the Golgi, where it is required for spermatocyte cytokinesis, but is dispensible for glue granule formation in salivary glands. PI4KII is localized to the Golgi and endosomes and is required during glue granule biogenesis, but is dispensible for spermatocyte cytokinesis. By contrast, PI4KIII α is essential and needed for generating pool of PI4P at the plasma membrane and cortical integrity during oogenesis. Since Fwd and PI4KII localize to the Golgi, we hypothesized that these enzymes might have overlapping functions. Thus, the main objective of our study was to investigate potential redundancy of Fwd and PI4KII in *Drosophila* cells. For our experiments, we used a combination of *Drosophila* genetic tools, immunohistochemical methods and confocal imaging. We found that GFP-Fwd and endogenous or mCherry-tagged PI4KII localize near each other at the Golgi in salivary gland cells and spermatocytes. In fwd PI4KII double mutants, which die as second instar larvae, the Golgi appear smaller and clathrin adaptor AP-1 puncta are not always associated with each Golgi body in salivary gland cells. Double knockdown of fwd and PI4KII by RNAi in *Drosophila* S2 cells displaced a fluorescent PI4P marker (YFP-PH-FAPP) from the Golgi into the cytoplasm, indicating that the two PI4Ks are redundant for synthesizing Golgi PI4P. In addition, fwd PI4KII double mutants showed severe Golgi phenotypes in egg chambers. Moreover, nurse cell membranes, which were not affected in fwd and PI4KII single mutant ovaries, appeared thinner and discontinuous in fwd PI4KII double mutant germ line clones, revealing the redundant roles of these PI4KII enzymes in plasma membrane stability. These results suggest that Fwd and PI4KII together might affect processes controlled by PI4KIII α .

P2004**Board Number: B522****E-cadherin apical sorting by a p200/AP-1/clathrin/RAB-11 pathway during morphogenesis.**G. Gillard¹, M. Shafaq-Zadah², O. Nicolle¹, G. Michaux¹;¹CNRS, IGDR, Rennes, France, ²INSERM U1143-CNRS UMR 3666, Institut Curie, Centre de Recherche, Paris cedex 05, France

Membrane traffic can be divided in two essential steps: sorting between cargos and transport to various destinations. In epithelial cells, a specific sorting event is required to differentially traffic apical and basolateral proteins; while basolateral sorting has been extensively studied, apical sorting mechanisms are still elusive. To characterise apical sorting *in vivo* we choose to examine the apical delivery of E-cadherin (E-cad) in *C. elegans* epidermal cells. A systematic RNAi screen designed to find genes required for the strict E-cad apical localisation identified clathrin, the clathrin adaptor AP-1 and p200/SOAP-1, a physical AP-1 interactor; their loss induced a basolateral localisation of E-cad while the loss of Rab5, Rab11 or the exocyst lead to intracellular accumulation. This phenotype was specific to E-cad since other apical markers and the apico-basal diffusion barrier were unaffected in the epidermis. Surprisingly glycosphingolipids were not required in that process, likely ruling out a role for lipid rafts, the best known apical sorting mechanism, in E-cad apical sorting. We further found that SOAP-1 controls AP-1 localisation which is itself required for Rab11 subapical localisation. Furthermore Rab11 depletion induced a strong loss of the apical E-cad pool. We concluded that SOAP-1/AP-1 control E-cad sorting upstream of its apical delivery via Rab11 endosomes in the biosynthetic and/or recycling pathways. This function of AP-1 in E-cad apical delivery is essential during embryonic morphogenesis, a process controlled by junction rearrangement and driven by acto-myosin contractions: we found that the E-cad basolateral accumulation was correlated in time and space with a strong loss of cell-cell adhesion and a dramatic loss of actin organisation. We therefore propose that a molecular pathway including SOAP-1, AP-1 and clathrin acts upstream of Rab11 to apically deliver E-cadherin in *C. elegans* epidermal cells and control cell-cell adhesion rearrangement during morphogenesis.

P2005**Board Number: B523****Plasma membrane trafficking mediated by a novel polybasic Golgi export signal in the reovirus p14 FAST protein is mediated by Rab11 and AP1.**H. Parmar¹, C. Barry¹, F. Kai¹, R. Duncan²;¹Microbiology and Immunology, Dalhousie University, Halifax, NS, ²Dalhousie University, Halifax, NS

The sorting and trafficking of integral membrane proteins to the plasma membrane is essential for proper cellular function. Our understanding of the pathways and signals involved in regulating this process is incomplete, particularly as it relates to Golgi export to the plasma membrane. The reovirus

fusion-associated small transmembrane (FAST) proteins traffic through the ER-Golgi pathway to the plasma membrane where they cause cell-cell membrane fusion. We undertook an analysis of factors regulating plasma membrane trafficking of the p14 FAST protein. Results indicate that a polybasic motif (PBM) in the cytosolic tail of p14 functions as a novel autonomous Golgi export signal. Alanine substitution of the p14 PBM led to loss of p14 plasma membrane localization and accumulation of p14 in the Golgi complex. Extensive mutagenic analysis indicated that a minimum of three basic residues are required for efficient Golgi export, and that the PBM exerts differential trafficking effects depending on its membrane proximity. Moreover, insertion of the PBM into a Golgi resident protein mediated protein export to the plasma membrane, indicating the p14 PBM is an autonomous Golgi export signal. Co-immunoprecipitation (Co-IP) indicated p14 interacts with activated Rab11 in a PBM-dependent manner, and RNAi approaches revealed Rab11 and adaptor protein 1 (AP1) are both required for efficient p14 trafficking to the plasma membrane. This is the first indication that interaction of activated Rab11 with membrane cargo can sort membrane cargo to AP1-coated vesicles for plasma membrane trafficking, and in the case of p14 this is mediated by a novel tri-basic Golgi export signal.

P2006

Board Number: B524

Role of tetanus neurotoxin insensitive vesicle-associated membrane protein in membrane microdomains transport and homeostasis.

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Biological membranes in eukaryotes contain a large variety of proteins and lipids often distributed in microdomains in plasma membrane and endomembranes. Molecular mechanisms responsible for the transport and the organization of these membrane microdomains along the secretory pathway still remain elusive. Here we show that vesicular SNARE TI-VAMP/VAMP7 plays a major role in membrane microdomains composition and transport. We found that the transport of exogenous and endogenous GPI-anchored proteins was altered in fibroblasts isolated from VAMP7-knockout mice. Furthermore, disassembly and reformation of the Golgi apparatus induced by Brefeldin A treatment and washout were impaired in VAMP7-depleted cells, suggesting that loss of VAMP7 expression alters biochemical properties and dynamics of the Golgi apparatus. In addition, lipid profiles from these knockout cells indicated a defect in glycosphingolipids homeostasis. We conclude that VAMP7 is required for effective transport of GPI-anchored proteins to cell surface and that VAMP7-dependent transport contributes to both sphingolipids and Golgi homeostasis.

P2007**Board Number: B525****Microtubule regulation of insulin packaging and secretion in pancreatic β -cells.**X. Zhu¹, R. Hu¹, D. Yampolsky¹, M. Brissova¹, R. Stein¹, A.C. Power¹, G. Gu¹, I. Kaverina²;¹Vanderbilt University medical center, Nashville, TN, ²Cell and Developmental Biology, Vanderbilt University Medical Center, Nashville, TN

Glucose-stimulated insulin secretion (GSIS) from pancreatic β cells maintains glucose homeostasis. The prevailing hypothesis is that insulin secretion by β cells is the result of microtubule-dependent transport, which delivers insulin granules to exocytic sites. In contrast to this common notion, we report that microtubules (MTs) regulate insulin secretion by restraining excessive delivery of insulin granules to the plasma membrane. Using high- and super-resolution microscopy on intact islets, combined with GSIS detection, we found that MTs in functional β cells emerge from the Golgi complex and form a meshwork-like network that anchors insulin granules. High glucose enhances MT dynamic turnover resulting in a less dense network, which is necessary for GSIS. Importantly, the density of MT network is significantly greater in dysfunctional β cells of diabetic mice compared to normal mice. Furthermore, we show that MT depolymerization facilitates delivery of insulin granules to the cell membrane and dramatically enhances GSIS, suggesting that dense MTs withhold granules away from secretion sites, and that glucose induces dynamic MT turnover and makes granules available for GSIS. Moreover, our data indicate that while insulin secretion is facilitated w/o MTs, proinsulin packaging into the granules at the TGN is retarded due to slow proinsulin sorting for entry. These results demonstrate that MTs act as a cellular “rheostat” for precisely metered insulin secretion, which is tuned according to physiological needs by modulating MT density and dynamics. Currently, we are in progress of developing a computational model, which will allow us to simulate relative contributions of distinct MT-dependent mechanisms to balanced insulin secretion.

P2008**Board Number: B526****Formin-mediated traffic control: Involvement of RhoA-mDia1 pathway in regulating the movements of secretory vesicles.**Z. Lieu¹, N. Flores-Rodriguez¹, S. Miserey-Lenkei², A. Mogilner³, A.D. Bershadsky^{1,4};¹Mechanobiology Institute, National University of Singapore, Singapore, Singapore, ²Institut Curie-Centre National de la Recherche Scientifique, Paris, France, ³Department of Biology, Courant Institute of Mathematical Sciences, New York, NY, ⁴Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel

The trafficking of secretory vesicles along microtubule track mediates the transport of cargo proteins or lipids from one intracellular compartment to another. While microtubule-based motor proteins and their associated regulatory machinery are known to be involved in regulating the mobility of secretory

vesicles along microtubule, the role of the actin cytoskeleton, in particular, the formin family of actin nucleators remains poorly understood. Using the secretory cargo, monocyte chemoattractant protein 1 (also known as CCL2), we showed that formin mDia1 and its activator RhoA are involved in regulating the movements of CCL2 vesicles along microtubules. Increasing the activity of RhoA using lysophosphatidic acid or expression of constitutively active RhoA significantly reduces the proportion of moving vesicles, the average anterograde velocity and the total vesicle displacement. These effects of RhoA were shown to be mediated by its immediate downstream target mDia1, a potent activator of actin filament nucleation and elongation. The overexpression of constitutively active mDia1 mutant was by itself sufficient to reduce the mobility of CCL2 vesicles to the levels similar to those observed in RhoA activated cells, suggesting that increased actin polymerization mediated by activated mDia1 restricts the motion of CCL2 vesicles. Several lines of evidence suggest that mDia1 effects on the movements of CCL2 vesicles may be regulated by local actin polymerization rather than overall increase in F-actin in cells. First, live imaging observations demonstrated that formin mDia1, but not mDia2, is transiently localised to CCL2 vesicles. Second, immunoprecipitation experiments demonstrated that mDia1 interacts with the kinesin-1 heavy chain as well as with the components of dynactin complex. Finally, alterations of global F-actin level in cells using actin polymerization-affecting drugs did not result in significant changes in the motility of CCL2 vesicles, as compared to those produced in cells by active mDia1 or RhoA. Taken together, our results are consistent with the hypothesis that mDia1 regulates the kinesin-1-driven movements of secretory vesicles along microtubules by affecting local actin polymerization.

P2009

Board Number: B527

The Arf GEF GBF1 integrates inputs from its product, Arf4•GTP, and the Golgi influx of sensory membrane cargo to activate Arf4 in ciliary trafficking.

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One of the outstanding questions in Arf GTPases regulation is the role of protein cargo in their activation. In this study, we show that during ciliary membrane trafficking a functional complex exists between the sensory membrane cargo, rhodopsin, the cognate Arf, Arf4, and the Arf GEF GBF1, which regulates Arf4 activation. The *in situ* Proximity Ligation Assay (PLA) revealed that GBF1 interacts with rhodopsin, Arf4, and the Arf GAP ASAP1 at the photoreceptor Golgi/TGN. The GBF1 GEF activity was essential for the GBF1-rhodopsin-Arf4 interaction, since its selective inhibitor Golgicide A (GCA) caused a significant decrease in all but the GBF1-ASAP1 interactions. GCA specifically inhibited rhodopsin delivery to the cilia. However, in contrast to BFA, GCA had no effect on the Golgi morphology. The GBF1 GEF activity involves the catalytic Sec7 domain. GBF1 also contains conserved regulatory domains: a dimerization and cyclophilin binding (DCB), a homology upstream of Sec7 (HUS), and three homology downstream of Sec7 (HDS) domains. GST-pulldowns of purified rhodopsin were performed with DCB-HUS and Sec7-HDS1, with or without recombinant Arf4 pre-loaded with GTPγS or GDPβS. GST-DCB-HUS pulled down rhodopsin significantly better than GST-Sec7-HDS1, or GST alone, either in the presence or

absence of Arf4, demonstrating a direct rhodopsin-GBF1 interaction. Arf4•GTP preferentially interacted with DCB-HUS, whereas Arf4•GDP interacted with Sec7-HDS1. Thus, GBF1 combines a regulatory binding site within its DCB-HUS domain with a catalytic binding site within the Sec7 domain and acts both as a GEF and an effector of Arf4. The influx of rhodopsin into the Golgi/TGN and Arf4•GTP likely cooperatively activate GBF1 to increase the levels of Arf4•GTP at the Golgi/TGN and optimize budding conditions for ciliary-targeted post-TGN carriers. Notably, the Arf GEF GBF1 and the Arf GAP ASAP1 directly interact with each other, and with rhodopsin and Arf4•GTP, to synchronize Arf4 activation cycles during ciliary receptor trafficking.

P2010

Board Number: B528

Energy-Dependent Regulation of Clathrin Adaptors by the Arf-like GTPase (Arl1).

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Cellular homeostasis requires adequate levels of glucose. Cells starved for glucose, such as during ischemia, experience severe stress and a reduction in viability. Our lab has discovered an important membrane trafficking response to glucose starvation. During this response, plasma membrane proteins of various types are internalized and delivered to the lytic compartment of the cell, the yeast vacuole. Understanding the regulation of this pathway will be important for shedding light on how cells respond to energy stress. Key regulators of membrane trafficking are obvious candidates, such as Arf GTPases. Similar to Arf1, Arl1 can interact with the clathrin adaptor, Gga2, in its GTP-bound state. Arl1 may therefore be able to recruit clathrin adaptors *in vivo*, however, the role of Arl1 in membrane trafficking has not been well-characterized. These studies provide evidence for a role of Arl1 in the regulation of endosome to vacuole transport during glucose starvation. Using live-cell imaging and western blotting, we demonstrate that Arl1 is required for endosome to vacuole transport of plasma membrane (PM) cargoes during glucose starvation. Apart from proteins, another PM cargo that can be delivered to the vacuole or lysosomes to be degraded and used for ATP generation is lipids. We show that depletion of Arl1 in mammalian cells results in a reduction in lipid droplet size and number during glucose starvation. Similarly, we demonstrate that Arl1 is required for endosomal localization of clathrin adaptors during energy stress. We show that the adaptors themselves are required for endosome to vacuole delivery of PM proteins during glucose starvation. Additionally, using a permeabilized cell assay, we can add ATP and GTP to yeast spheroplasts and stimulate the recruitment of clathrin adaptors to puncta. Previously, we found that GTP synergizes with ATP to recruit adaptors. Our recent data shows that loss of ARL1 eliminates this GTP-ATP synergy. This suggests that Arl1 acts directly in the recruitment of clathrin adaptors, with the help of an unknown ATP sensor. Importantly, loss of ARL1 and subsequent endosome to vacuole transport during glucose starvation leads to reduced ATP recovery and reduced

cell viability. Lastly, we are working to see if the PM to vacuole shunt and the role of Arl1 during glucose starvation are conserved in mammalian cells. Cumulatively, these data point to an energy-dependent role for Arl1 in the regulation of clathrin adaptors at the endosomes and subsequent endosome to vacuole transport. This trafficking step is critical for cell survival during starvation. We propose that, through a mechanism similar to Arf1, Arl1 can recruit clathrin adaptors to the TGN and endosomes during energy stress to promote cell survival.

P2011

Board Number: B529

Mechanism of action of flippase Drs2p in modulating GTP hydrolysis of Arl1p.

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Small GTPase ADP-ribosylation factors are key regulators of membrane trafficking and their activities are determined by guanine nucleotide-binding status. Arl1p, an ARF-like protein, is responsible for multiple trafficking pathways at the Golgi. The GTP-hydrolysis of Arl1p is stimulated by its GTPase-activating protein Gcs1p, and binding with its effector Imh1p protects Arl1p from premature inactivation. However, the mechanism involved in the timing of Arl1p inactivation is unclear. Here, we demonstrate that another Arl1p effector, lipid flippase Drs2p, is required for Gcs1p-stimulated inactivation of Arl1p. Drs2p is known to be activated by Arl1p and is involved in vesicle formation through its ability to create membrane asymmetry. We found that the flippase activity of Drs2p is required for proper membrane targeting of Gcs1p in vivo. Through modification of the membrane environment, Drs2p promotes the affinity of Gcs1p for the Golgi where it binds to active Arl1p. Together, Imh1p and Drs2p modulate the activity of Gcs1p by timing its interaction with Arl1p, hence providing feedback regulation of Arl1p activity.

P2012

Board Number: B530

The role of phosphatidylserine flippase activity in lateral membrane organization.

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An asymmetric distribution of phospholipid species, particularly at the plasma membrane (PM), is a common feature of many eukaryotic cells. Establishment and maintenance of membrane asymmetry requires the activity of flippases that translocate phospholipid substrates from the luminal/extracellular leaflet to the cytosolic leaflet. While the role of flippases in the transverse segregation of phospholipids has been well established, little is known about the role of flippases in lateral membrane organization. The budding yeast PM is organized laterally into a patchwork of “compartments” formed through lipid-

lipid and lipid-protein interactions and marked by specific membrane proteins. In budding yeast there are five flippases (Dnf1p, Dnf2p, Dnf3p, Drs2p, and Neo1p) and there is suggestive evidence that the yeast flippase Drs2p, which has substrate specificity for the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE), may have a role in lateral membrane organization. Ergosterol, a core component of lateral membrane domains which normally localizes to the PM, accumulates in internal membranes in *drs2Δ* cells. Membrane proteins which partition into distinct membrane compartments (Pma1p and Can1p) also partially mislocalize to the vacuole in *drs2Δ* cells, further indicating loss of Drs2p flippase activity is disrupting lateral membrane organization. To determine if these defects are specifically due to loss of PS flip, two flippase constructs were used that lose (*drs2*-[QQ->GA]) or gain (DNF1 N550S) the ability to flip PS. *drs2*-[QQ->GA], despite retaining the ability to flip PE, is not able to suppress the ergosterol and membrane protein mislocalization phenotypes of *drs2Δ* cells. In contrast, DNF1 N550S, which gains the ability to flip PS in addition to its normal lysophospholipid substrates, is able to suppress the *drs2Δ* defects. These results suggest that PS flip by Drs2p plays an important functional role in the generation and maintenance of lateral membrane organization and provides new insight into how lateral domains are established in budding yeast.

P2013

Board Number: B531

Structure and function of the N-terminal domains of Sec7/BIG1/2 regulating Arf1-mediated trafficking from the trans-Golgi network.

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Export of trafficked proteins from the trans-Golgi network (TGN) is an essential function in all eukaryotes, controlled by the small GTPase Arf1. The proximal upstream regulator of Arf1 is the conserved Sec7 (yeast) / BIG1/2 (mammal) / Sec71 (fly) guanine nucleotide exchange factor (GEF), which activates Arf1 specifically at the TGN. This action initiates the terminal cascade of events at the Golgi leading to cargo sorting and vesicle formation with multiple different cargos and cargo adaptors.

Sec7 consists of a single well-characterized catalytic domain responsible for its GEF activity on Arf1, and six similarly sized domains, two N-terminal and four C-terminal to the GEF domain. We recently demonstrated that these latter six domains serve varying and essential functions both to localize Sec7 to its point of action and to regulate its activity negatively when unlocalized and positively when properly localized. Together, the regulatory domains of Sec7 tightly restrict its activity to the trans-Golgi network, ensuring proper maturation and targeting of all transported proteins. However, the mechanistic details of how each domain mediates its autoregulatory functions and its interactions with other proteins remain obscure.

Here, we present the 2.7 Å crystal structure of the two N-terminal regulatory domains of Sec7, representing the first structural model available for regions of Sec7 outside the catalytic domain. The structure identifies a pair of conserved surface regions, one in each domain, mutation of which affects

the activity of Sec7 on Arf1 both *in vitro* and *in vivo*. Paired with small-angle x-ray scattering and multi-angle light scattering data of a suite of Sec7 fragments, this structure provides insight into the nature of dimerization mediated by the N-terminal domains and into the relationship between the catalytic domain and the N-terminal domains.

P2014

Board Number: B532

Defining the assembly determinants and architecture of the yeast exocyst complex.

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The exocyst is a highly conserved, hetero-octameric protein complex, which is proposed to function at the tethering step of exocytosis in all eukaryotes. The classification of the exocyst as a multisubunit tethering complex (MTC) stems from its known interacting partners, polarized localization at the plasma membrane, and structural homology to other putative MTCs. The presence of 8 subunits begs the questions: why are so many subunits required for vesicle tethering and what are the contributions of each of these subunits to the overall structure of the complex? Additionally, are subunit or subcomplex dynamics a required feature of exocyst function? In order to answer these questions, it was critical that we accurately define the subunit connectivity within the endogenous exocyst complex. Using *Saccharomyces cerevisiae* as our model system, we purified intact exocyst complexes under physiological conditions. Regardless of the exocyst subunit used as purification handle, all 8 subunits are stoichiometric. In contrast to early studies of the exocyst, these complexes remained assembled over a wide range of pH and ionic strength conditions, suggesting greater intra-complex stability than previously expected. However, at higher extremes of pH and salt concentration, biochemically stable subcomplexes including Sec10-Sec15 and Sec3-Sec5-Sec6-Sec8 emerged. To further dissect these connections and because most of the exocyst subunits are encoded by essential genes, we used the auxin-inducible degradation (AID) system to selectively and rapidly degrade individual exocyst subunits and monitor the effects on complex assembly. Using the AID system, we identified critical subunits that are required for the overall assembly and for connecting modular subcomplexes. However, given the highly stable assembly of the complex, we hypothesize that any subunit dynamics or complex disassembly require additional factors. To address this hypothesis, we are using the AID system to identify which components within the post-golgi secretory pathway are required for the assembly and disassembly of the exocyst. By selectively depleting GTPases, myosin, and SNAREs and then purifying endogenous exocyst complexes, we will pinpoint the steps in the pathway when these complex rearrangements may occur. Finally, piecing together these subunit connections into the overall structure of the exocyst will provide clues as to how the subunits are poised to capture secretory vesicles. To this end, we visualized its structure for the first time using negative stain electron microscopy. 2D class

averaging reveals an elongated conical structure, and we are currently working to position the individual exocyst subunits within this overall structure.

P2015

Board Number: B533

Trafficking of the Kir2.1 potassium transporter is regulated by the ubiquitin ligase Rsp5 and a select subset of alpha-arrestins in yeast.

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To ensure optimal cell growth and survival, protein composition at the plasma membrane is tightly regulated, with both controlled protein internalization and selective protein targeting to the cell surface in response to environmental changes. To identify factors that regulate this selective protein trafficking, we developed a yeast expression system for the Kir2.1 potassium channel, which maintains potassium homeostasis in heart cells and is thereby critical for cardiac function. By monitoring the growth of Kir2.1 yeast on low potassium medium, Kir2.1 activity at the cell surface can be assessed. Using a high-throughput screening methodology we identified the ESCRTs and retromer complex as novel regulators of Kir2.1 trafficking. We now show that three specific alpha-arrestins – Ldb19/Art1, Aly1/Art6, and Aly2/Art3 – control Kir2.1 trafficking. In marked contrast to the well-described role for alpha-arrestins as endocytic adaptors, these alpha-arrestins promote Kir2.1 trafficking to the cell surface, increasing Kir2.1 activity at the plasma membrane and raising intracellular potassium levels. Consistent with the alpha-arrestin requirement, we also demonstrate that the ubiquitin ligase, Rsp5, and the protein phosphatase calcineurin regulate Kir2.1 plasma membrane targeting. Furthermore, by fusing a single chain antibody to Kir2.1 and employing live cell fluorescence microscopy, we can selectively monitor Kir2.1 cell surface localization or its intracellular distribution, thus providing a complementary tool to delineate the intracellular sorting pathway for this channel. Current work is focused on using the yeast model to define additional factors and regulatory elements required for alpha-arrestin-mediated trafficking of Kir2.1 to the cell surface. Together, these findings identify Kir2.1 as new alpha-arrestin substrate and lay the foundation to further define the molecular mechanisms governing alpha-arrestin-mediated intracellular sorting.

P2016**Board Number: B534****CLATHRIN ADAPTOR AP-1 CONTROL BASOLATERAL SORTING OF THE CHLORIDE CHANNEL 2 THROUGH A SOLE DILEUCINE MOTIF.**E. de la Fuente-Ortega*¹, D. Gravotta*², C. Lagos³, A. Perez-Bay², G. Lehmann², I. Benedicto², E. Rodriguez-Boulan²;¹Department of Biomedical Sciences, Faculty of Medicine, Universidad Católica del Norte, Coquimbo, Chile, ²Department of Ophthalmology, Dyson Vision Research Institute, Weill Medical College of Cornell University, New York, NY, ³Department of Pharmacy, Faculty of Chemistry, Pontificia Universidad Católica de Chile, Santiago, Chile

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Voltage-regulated chloride channel 2 (ClC2) is a polytopic protein ubiquitously expressed in the basolateral membrane of many epithelial cells that function in the regulation of cell volume, ion transport and acid-base balance. As for most chloride channels, the molecular signals and cellular machinery that determine the subcellular distribution of ClC2 are poorly understood. Here, we combined *in silico* modeling, biochemical and cellular approaches to study the mechanisms that control the basolateral localization of ClC2 in polarized MDCK cells. ClC2 exhibit three putative dileucine motifs in its C-terminus, two in cystathionine β -synthase domain 1 (CBS1) and a third motif, previously implicated in basolateral sorting, within the CBS2. Our *in silico* model of ClC2 however revealed that CBS2-motif, TIFS⁸¹²LL, was buried within the dimeric structure of the channel and inaccessible, while CBS1-motifs, ESMI⁶²³LL and QVVA⁶³⁵LL, displayed a higher exposure toward the solvent. Consistently yeast three hybrid assays and *in silico* modeling of AP-1 demonstrated that ESMI⁶²³LL favorably interacted with a highly conserved pocket in the gamma-sigma1A hemicomplex of AP-1. Single alanine mutagenesis of these motifs and trafficking analysis of these mutants in MDCK cells demonstrated that ESMI⁶²³LL acted as a sole basolateral sorting signal. Single and double knockdown experiments indicated that clathrin adaptors AP-1A and AP-1B were individually necessary and sufficient for basolateral localization of ClC2. Our study constitutes the first structure-function analysis of a dileucine-based basolateral sorting mechanism in epithelial cells. Supported by NIH grants EY08538 and EY022165 to ERB, a Chilean government fellowship to EdIF and a PEW Latin American postdoctoral fellowship to GL.

P2017**Board Number: B535****2-deoxyglucose induces α -arrestin-dependent trafficking of hexose transporters via signaling through the Snf1/PP1 phosphatase pathway.**A.F. O'Donnell^{1,2}, R.R. McCartney³, D.G. Chandrashekarappa³, B.B. Zhang³, J.W. Thorner⁴, M.C. Schmidt³;¹Cell Biology, Univ Pittsburgh, Pittsburgh, PA, ²Dept. of Molecular and Cell Biology, University of California at Berkeley, Berkeley, CA, ³Dept. of Microbiology and Molecular Genetics, University of Pittsburgh, Pittsburgh, PA, ⁴Dept. of Molecular and Cell Biology, University of California, Berkeley,

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2-deoxyglucose (2DG), a non-metabolizable analogue of glucose thought to block glycolysis, prevents cancerous growth in animal models and in human tumor cell lines. Despite its widespread use and important clinical applications, the mechanism of 2DG action remains unclear. Our study demonstrates that 2DG is a potent inhibitor of yeast cell growth, even when cells are grown in the presence of abundant glucose. Using a comprehensive high-throughput screen, we demonstrate that over-expression of either of the two low-affinity hexose transporters – Hxt1 and Hxt3 – can suppress yeast sensitivity to 2DG. Furthermore, we find that in response to 2DG addition, these transporters are rapidly internalized and trafficked to the vacuole for degradation. 2DG-induced internalization of Hxt1 and Hxt3 requires Rod1/Art4 and Rog3/Art7, members of the alpha-arrestin family of trafficking adaptors. Consistent with a role for Rod1 and Rog3 in mediating cellular response to 2DG, cells lacking these alpha-arrestins are resistant to 2DG and 2DG fails to trigger Hxt1 and Hxt3 internalization and trafficking to the vacuole. Importantly, Hxt1 and Hxt3 internalization in response to 2DG is regulated by the Snf1 protein kinase, the ortholog of which is mammalian AMPK. In the presence of 2DG, Snf1 becomes moderately activated, as indicated by phosphorylation on its activation loop, and selectively phosphorylates Rod1 and Rog3, while other known Snf1 substrates, including the transcriptional repressor Mig1, are not phosphorylated under these conditions. Addition of 2DG not only increases the Rod1 and Rog3 phosphorylation, but dramatically increases the ubiquitination of these alpha-arrestins. We further show that binding to the ubiquitin ligase Rsp5 is required for Rod1- and Rog3-mediated trafficking of the Hxts and induction of 2DG sensitivity. However, while phosphorylation of Rod1 and Rog3 may facilitate their ubiquitination it is not required for ubiquitination as in the absence of Snf1, both alpha-arrestins are ubiquitinated and able to stimulate Hxt internalization. Based on these findings we propose a mechanism for 2DG-induced toxicity in yeast whereby Snf1 becomes activated, triggering phosphorylation and subsequent ubiquitination of the alpha-arrestins Rod1 and Rog3, which in turn induces Hxt1 and Hxt3 endocytosis and trafficking to the vacuole. By reducing glucose transporter levels at the cell surface, 2DG causes a glucose starvation response, even when cells are grown in a glucose-rich environment. Our findings may shed light on the long elusive mechanism of 2DG in inducing cancer cell death.

P2018

Board Number: B536

In vivo imaging reveals that restricted exocytic trafficking drives cell shape changes required for intercalation during chondrocyte stacking.

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Mechanisms that control cell shape changes and 3D tissue organization play a crucial role in morphogenesis. A striking example occurs during vertebrate cartilage development, when clusters of

small chondrocytes transition to stereotypical rows of elongated cells that build intricate skeletal structures by a process called stacking. Although this process has been observed for nearly a century, the cellular programs that execute chondrocyte shape changes during stacking are largely unknown. Here, using imaging, genetic and cell biology approaches we identify Erc1 as a novel component of the vesicle trafficking pathway that regulates chondrocyte cell elongation *in vivo*.

During stacking, Erc1-deficient cells become hyper-elongated but fail to expand in width, leading to chondrocyte cell death and craniofacial dysmorphology. We show that Erc1 is required to promote orientation of microtubules, and Erc1 depletion results in aberrant microtubule localization at polarized cortical regions of intercalating chondrocytes. These changes in microtubule polarity do not drive cell shape change in an active mode, but instead correlate with vesicle trafficking defects. We observed that this mechanism is engaged exclusively during chondrocyte cell shape changes.

Erc1 depletion leads to accumulation of vesicular structures at the chondrocyte cell cortex as well as the intracellular retention of glycosylated ECM components, suggesting impaired exocytosis. We demonstrate by *in vivo* mosaic analysis and *in situ* imaging in the developing cartilage that Rab8 and Kinesin-1 contribute to Erc1-dependent chondrocyte cell shape changes. Our study identifies for the first time a cellular mechanism of chondrocyte cell shape regulation that drives stacking and organ morphogenesis while utilizing microtubule polarity and vesicle trafficking machinery.

P2019

Board Number: B537

Lipid Binding by Osh4p, an OSBP homologue, is Required for Polarized Exocytosis.

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Polarized exocytosis is an essential cellular process required for diverse events such as polarized cell growth and secretion, and cell migration. The seven-member Osh protein family, the *S. cerevisiae* homologues of the mammalian oxysterol-binding protein (OSBP) family, is required for polarized exocytosis (Alfaro et al., *Traffic*, 2011). Within the Osh protein family, Osh4p (Kes1p) is known to bind and transfer specific lipid species between membranes *in vitro*, however whether lipid binding and transfer by Osh4p is an essential activity *in vivo* has remained elusive (de St. Jean et al., *JCB*, 2012; Georgiev et al., *Traffic*, 2011). Two questions regarding Osh4p function are whether Osh4p regulates non-polarized exocytosis in addition to polarized exocytosis and whether lipid binding by Osh4p regulates its role in polarized exocytosis. First, we show data excluding a role for Osh4p in non-polarized exocytosis as marked by invertase secretion. Further we show that the dominant lethality of the osh4Y97F allele, which encodes a sterol binding null Osh4p, is dependent on the ability of Osh4p to bind phosphatidylinositol-4-phosphate (PI4P) and that Osh4p must be able to bind both sterols and PI4P for polarized exocytosis, marked by Bgl2p, to occur. These studies show that Osh4p is dependent on its

lipid binding activity to function in polarized exocytosis, with PI4P serving as a positive regulator of Osh4p function. These results suggest that other Osh or OSBP family members will be shown to regulate polarized exocytosis or other membrane-membrane contact sites in a lipid dependent manner.

P2020

Board Number: B538

GGA3 modulates the trafficking of G protein-coupled receptors via direct interaction.

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The molecular mechanisms underlying the cell surface transport of newly synthesized G-protein-coupled receptors (GPCRs) remain poorly defined. Here, we determined the role of GGA3 [Golgi-localized, gamma-adaptin ear domain homology, ADP ribosylation factor (ARF)-binding protein 3] in the cell surface targeting of alpha2B-adrenergic receptor (AR), a prototypic GPCR. As an adaptor protein for clathrin-coated vesicles, GGA3 modulates the transport between the trans-Golgi network (TGN) and endosomes and its function is tightly controlled by specific interactions of the VHS domain with the acidic LL motifs presented in the C-terminus of cargo molecules. We found that siRNA-mediated depletion of GGA3 significantly attenuated the cell surface expression of alpha2B-AR and arrested the receptor in the Golgi/TGN compartment. Consistent with the reduction in the cell surface expression, the function of alpha2B-AR measured as MAPK activation was markedly inhibited in response to agonist stimulation in cells transfected with GGA3 siRNA. More interestingly, although alpha2B-AR does not possess any acidic LL motifs, it strongly interacted with GGA3 and the interaction domains were subsequently mapped to the VHS domain of GGA3 and the third intracellular loop of the receptor. These data demonstrate an important function for GGA proteins in the Golgi-to-plasma membrane transport of GPCRs which is likely mediated through a non-conventional mechanism.

P2021**Board Number: B539****A phosphorylation-dependent sorting motif restricts presenilin 2 to late endosomes/lysosomes providing a basis for substrate specificity.**R. Sannerud¹, C. Esselens¹, L. Rochin², A.K. Tharkeshwar¹, G. De Baets³, P. Ejsmont¹, V. Baert¹, W. Vermeire¹, S. Munck⁴, J. Schymkowitz³, F. Rousseau³, G. Van Niel², W. Annaert⁵;¹Center for the Biology of Disease, VIB / KULeuven, Leuven, Belgium, ²Subcellular Structure and cellular Dynamics, Institut Curie, Paris, France, ³Switch Laboratory, VIB / KULeuven, Leuven, Belgium, ⁴LiMoNe, VIB, Leuven, Belgium, ⁵KU Leuven and VIB Center for the Biology of Disease, Campus Gasthuisberg, POB 602, OandN4, Room 7.159, Laboratory of Membrane Trafficking, Center for Human Genetics, Leuven, Belgium

Regulated intramembrane proteolysis (RIP) abrogates or initiates downstream signalling by cleaving substrate proteins within their transmembrane domains. γ -Secretase governs such mechanism by targeting >100 type I proteins, including amyloid precursor protein (APP) and Notch. Its activity is confined to a complex comprising presenilin (PSEN), nicastrin, APH-1 and PEN-2. The two homologous (PSEN1&2) and several APH-1 isoforms give rise to complex heterogeneity within the cell of which the functional relevance is largely unexplored. Using biochemical and confocal approaches, we now reveal that, as opposed to the broad distribution of PSEN1/ γ -secretase, PSEN2/ γ -secretase is essentially confined to late endosomes/lysosomes in different cell types including primary hippocampal neurons. Domain swapping and mutational analysis identified a unique N-terminal motif in PSEN2 that directs PSEN2/ γ -secretase to late endosomes through a direct phosphorylation-dependent interaction with the AP-1 adaptor complex. This restricted localisation limits substrate availability for PSEN2/ γ -secretase, providing the first cell biological basis and relevance of complex heterogeneity.

P2022**Board Number: B540****Palmitoylated Ras-driven MAPK signaling and transformation is spatially regulated by plasma membrane microdomains.**J.V. Michael¹, J.G. Wurtzel¹, L.E. Goldfinger^{1,2};¹Department of Anatomy and Cell Biology and the Sol Sherry Thrombosis Research Center, Temple University School of Medicine, Philadelphia, PA, ²Cancer Biology Program, Fox Chase Cancer Center, Philadelphia, PA

This study addresses the spatiotemporal regulation of Ras/MAP kinase (MAPK) signaling and mitogenic and tumorigenic outcomes. Somatic mutations in the Ras-superfamily of small GTPases are highly prevalent in cancers. Activating mutations in Ras (e.g., H-Ras) cause uncontrolled signaling through the MAPK pathway via activation of Raf kinase. After recruitment to the plasma membrane Raf propagates a mitogenic cascade by phosphorylating MEK, leading to ERK-mediated transcription. H-Ras also has been

shown to support induction of tumor angiogenesis. Related-Ras (R-Ras) has a nearly identical sequence with H-Ras, but is unable to drive MAPK signaling. R-Ras is anti-angiogenic and weakly mitogenic, but is not tumorigenic, and activating mutations have not been linked with human cancers. R-Ras can bind Raf *in vitro*, but does not bind *in vivo*. These paralogues have identical effector-binding regions, and differ primarily in the C-terminal domains, which harbor sequence-specific target sites for lipid modifications that direct targeting to distinct plasma membrane (PM) microdomains: H-Ras to lipid raft/non-raft borders, R-Ras to rafts. We hypothesized that the lateral spatial segregation at the PM regulates effector interactions, downstream signaling, and functional effects. We used targeting domain (tD) swap mutants between H- and R-Ras, and assessed the signaling, cellular and tumorigenic effects. Activated R-Ras harboring the H-Ras tD (R-Ras-tH), stably expressed in NIH3T3 fibroblasts, interacted with Raf and propagated MAPK signaling through MEK and ERK phosphorylation in the absence of growth factors. Furthermore, R-Ras-tH stimulated cell proliferation under these conditions, and supported anchorage-independent growth in soft agar, similar to activated H-Ras, indicating induction of a transformed phenotype *in vitro* by the R-Ras-tH. Conversely, H-Ras harboring the R-Ras tD (H-Ras-tR), did not stimulate MEK or ERK phosphorylation, or proliferation, and these cells displayed significantly reduced colony formation in soft agar compared with active H-Ras cells. Thus, access to Raf at the plasma membrane is a critical step for Ras-driven mitogenic signaling and transformation. A tumor xenograft model showed that R-Ras-tH promoted tumor formation whereas R-Ras cells yielded few tumors; however, R-Ras-tH tumors showed no neovascularization, which blunted tumor progression. Both H-Ras and H-Ras-tR xenografts formed very large, highly vascularized tumors. Thus, factors outside of the tDs affect tumor progression distinct from *in vitro* transformation, as H-Ras-tR appears to drive tumorigenesis through a MAPK-independent pathway *in vivo*. Together, these results indicate that the angiogenic function of Ras isoforms is independent of PM microdomain localization.

P2023

Board Number: B541

Cytosolic N-terminal proline-rich domains of MAL2 confer specificity to protein trafficking in polarized hepatic WIF-B cells.

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Epithelial cells rely on the formation of tight junctions at the plasma membrane to create two major membrane domains, the apical (AP) and basolateral (BL), which separates the external from the internal environments. The establishment and maintenance of these membrane domains are driven by a highly regulated protein trafficking pathway that selectively deliver newly-synthesized AP or BL resident proteins to their respective membrane domains. Polarized hepatocytes use the indirect route to deliver newly-synthesized apical resident proteins from the trans-Golgi network (TGN) to the BL from which they are selectively endocytosed into basolateral early endosomes (BLEE), transcytosed to the subapical compartment (SAC) and finally delivered to the AP. Myelin and lymphocyte protein 2 (MAL2) has been

identified as an important apical protein trafficking regulator of the indirect route in polarized WIF-B cells in at least two stages: from the TGN to the BLEE and from the BLEE to the SAC. More recently, we have determined that MAL2 also regulates constitutive secretion from the TGN to the BL. Our hypothesis is that MAL2 coordinates with multiple binding partners thereby conferring specificity to cargo selection and vesicle targeting to distinct locations. Once the cargo is packaged into discrete vesicles, we propose that sorting specificity may be conferred by interactions of distinct MAL2 binding partners (e.g. rab17 and Ser/Thr kinase 16) with the divergent MAL2 N-terminal domain. Thus, we predicted that the MAL2 cytoplasmic domains mediate interactions with other proteins or lipids that in turn confer specificity to apical sorting/targeting. Interestingly, the MAL2 N-terminus encodes VPPPP and FPAP sequences that resemble the F/L/W/YPPPP recognition sites for EVH1 (enabled, VASP, homology1) motifs present in Ena/VASP proteins, suggesting a possible role of MAL2 in actin dynamics. The N-terminus also encodes a predicted phosphorylated site (ser 17) that may be important in regulating protein-protein interactions. To test our hypothesis, we will overexpress C-terminal FLAG-tagged MAL2 constructs using recombinant adenoviruses in WIF-B cells to allow their detection relative to endogenous MAL2. We will change the VPPPP and FPAP motifs to alanines either alone or together, deleting them individually or together and adding them to the MAL N-terminal sequence. We will also mutate ser 17 to ala or asn (a phospho-mimetic) and analyze their distribution and function in protein trafficking. Finally, we will examine if the C-terminal LRRW in MAL2 mediates lipid association like in MAL. Such careful and rigorous analysis will identify which (if any) of the domains confer specificity of MAL2 binding interactions and subsequent vesicle targeting.

P2024

Board Number: B542

Specific targeting/cellular localization of HCMV tegument protein pUL71 plays a critical role in production of infectious virus particles.

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The human cytomegalovirus (HCMV), also known as human herpesvirus-5, is a common virus that can cause life-threatening infection in immunocompromised patients. The HCMV UL71 gene product, a component of the viral tegument layer, is a palmitoylated, oligomeric protein that plays a critical role in secondary envelopment of viral particles. Although previously described as non-essential, deletion of pUL71, mutation of palmitoylation sites, or deletion of oligomerization sequences severely impairs production of infectious virus. To explore involvement of pUL71 in viral assembly, we generated a series of C-terminal truncation mutants. Co-localization studies in transfected cells showed that pUL71 has strong preferential localization to the trans-Golgi compartment. At the position 23-26AA of pUL71 we identified a YxxΦ sorting signal responsible for interaction with the μ subunit of Adaptor Protein complex. The mutation of YVLL sequence of pUL71 to alanines (mutant 23A) exhibited a strikingly different phenotype as compared to that of the wild type (WT) protein, with characteristic plasma membrane localization and decreased presence at the Golgi apparatus. Considering the close proximity

of the mutations to the palmitoylation sites of pUL71 we tested incorporation of modified palmitic acid in transiently expressed WT and 23A mutant. The lack of differences in palmitoylation showed that the phenotype of the 23A mutant was caused by the replacement of the YVLL with AAAA. To elucidate the functional significance of the YxxΦ motif in assembly of the virus, a recombinant mutant virus was created. The 23A mutant virus showed extremely slow growth and infectivity rate. The electron microscopy analysis of cells infected with the 23A mutant virus showed accumulation of empty capsids and partially tegumented sub-viral particles in the cytoplasm. Our results indicate the existence of a new functional motif in pUL71 distinct from previously described palmitoylation and oligomerization signals, and one that is vital for localization of pUL71 and its function in HCMV assembly. It is attractive to speculate that tegument protein pUL71 may cycle between trans-Golgi, TGN, endosomes and plasma membrane, compartments that are dramatically modified during the process of infection, in a similar manner to that of envelope glycoproteins of HCMV.

P2025

Board Number: B543

Molecular basis of the phenotypic heterogeneity in congenital sucrase-isomaltase deficiency.

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The etiology of chronic, severe and common gastrointestinal symptoms (GI) such as osmotic diarrhea and abdominal pain is oftentimes due to carbohydrate malabsorption triggered by reduced levels of disaccharidase activity at the apical membrane of the enterocytes. Congenital sucrase-isomaltase deficiency (CSID) is a prominent form of carbohydrate malabsorption disorders that is characterized by altered trafficking and functioning of sucrase-isomaltase (SI), the most active intestinal disaccharidase. CSID is commonly detected as a recessive inborn defect that is elicited by mutations in the coding region of SI. Biochemical and cellular analyses of several mutations have established the phenotypes concept of CSID according to the cellular location, intracellular processing and function of SI (for a review see Naim et al., JPGN 2012). While the clinical presentation of CSID in most reported cases is severe, it is worthwhile to investigate whether a mild to severe spectrum of GI symptoms exists in heterozygotes or other less severe genotypes that would occur at a higher frequency in the general population. Here, we investigated the molecular pathogenesis of 14 naturally occurring mutations of the SI gene in patients with a prior diagnosis of CSID and varying enzyme activity levels. (Uhrich et al, JPGN 2012). The mutations were introduced into the cDNA of wild type SI by site-directed mutagenesis and expressed in COS-1 cells. Analysis of the structural and biosynthetic features of the mutants, their cellular localization and enzymatic function revealed three major categories of CSID. The first category is comprised of mutants that are trafficked and processed in a fashion similar to wild type SI and with mostly normal, but varying, levels of enzymatic activity. The second class of mutants revealed delayed trafficking and maturation kinetics as well as reduced enzymatic activities. Finally, the third group of mutants is entirely

transport-incompetent and persists in the ER as mannose-rich, immature and enzymatically inactive proteins that are ultimately degraded, presumably in the proteasome. Notably, the expression of active, wild-type SI proteins is negatively impacted by malformed SI mutants in heterozygotes. The current study supports the existence of heterogeneous forms of CSID that vary in their degree of enzyme activity and clinical severity. These forms are primarily generated by the interaction of two mutants of SI or a mutant SI with its wild type counterpart. Furthermore, variations in the degree of severity are dictated by the malformed structure of SI and its trafficking rate, which may vary in heterozygotes.

P2026

Board Number: B544

Mx1, a large GTPase involved in intracellular transport.

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The plasma membrane (PM) of polarized epithelial cells can be subdivided into an apical and basolateral domain, both separated by tight junctions. Targeting cargo molecules to their correct site of delivery is provided by a complex intracellular sorting and transport machinery. The analysis of post-TGN (Trans Golgi Network) exocytic carriers for new components involved in apical trafficking revealed the presence of the large GTPase Mx1 on these vesicles in Madin Darby Canine Kidney (MDCK) cells. Mx1 is known so far as an antiviral factor induced by the Interferon system after viral infections. However Mx1 is constitutively expressed in MDCK cells. Interestingly, the intracellular amount of Mx1 increases during early days of epithelial polarization. We could see that this dynamin-related protein associates with cargo vesicles guided to the apical PM. Furthermore, siRNA mediated knockdown of Mx1 significantly reduces the apical delivery of both soluble and membrane proteins. Our recent data show an interaction of this GTPase with the tubulin and actin cytoskeleton in the subapical region of MDCK cells. Fluorescence microscopy data indicate that Mx1 also colocalizes with different motor proteins.

P2027

Board Number: B545

The Role of Sec3p in Secretory Vesicles Targeting and Exocyst Complex Assembly.

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During membrane trafficking, vesicular carriers are transported and tethered to their cognate acceptor compartments before SNARE-mediated membrane fusion. The exocyst complex was thought to target and tether post-Golgi secretory vesicles to the plasma membrane during exocytosis. However, no definitive experimental evidence is available to support this notion. We have developed an ectopic targeting assay in yeast, in which each of the eight exocyst subunits was expressed on the surface of

mitochondria. We find that most of the exocyst subunits were able to recruit the other members of the complex there, and mis-targeting of the exocyst led to secretion defects in cells. On the other hand, Sec3p, but not other members of the exocyst, is capable of recruiting secretory vesicles. Our assay also suggests that both cytosolic diffusion and cytoskeleton-based transport mediate the recruitment of exocyst subunits and secretory vesicles during exocytosis. In addition, the Rab GTPase Sec4p and its guanine nucleotide exchange factor Sec2p regulate the assembly of the exocyst complex. Our study helps to establish the role of the exocyst subunits in tethering and allows the investigation of the mechanisms that regulate vesicle tethering during exocytosis.

Endocytic Trafficking 3

P2028

Board Number: B546

A flow cytometry based mutagenesis screen to identify novel endocytic factors.

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Clathrin-mediated endocytosis involves the coordination of many proteins to uptake nutrients, and respond to extracellular signals. With the plethora of proteins that need to be internalized from the plasma membrane, and the needs of the endocytic machinery to respond to many types of signals, it is unlikely we have identified all the factors involved in endocytosis. Using *Saccharomyces cerevisiae*, we performed an EMS mutagenesis screen to identify novel genes that contribute to endocytosis using an endogenous cargo, the methionine permease protein Mup1 to measure endocytic activity. Mup1 localization was visualized and quantified using a pHluorin (pHI) tag, which is a pH-sensitive GFP variant that is quenched upon delivery to the acidic environment of the vacuole lumen. In cells grown in low methionine conditions, Mup1 accumulates at the plasma membrane, whereas Mup1 is internalized and trafficked to the vacuole in high levels of methionine. We used flow cytometry to enrich for mutagenized cells with elevated fluorescence, where Mup1-pHI failed to traffic to the vacuole following addition of methionine. Over 1000 positive colonies were secondarily screened by microscopy. Defects in endocytosis and trafficking of Mup1-pHI were confirmed in 256 mutants, and these were categorized according to Mup1 subcellular localization. Eight recessive mutants with general endocytic defects (defined as having defects for all endocytic cargos thus far tested) were selected for further characterization, and two of these mutants are truncations of the known endocytic genes, SLA1 and SLA2. We are currently identifying the remaining general endocytic mutants using whole genome sequencing. As expected, some mutants with specific defects in the endocytosis of Mup1 are complemented by LDB19/ART1, a protein known to contribute to Mup1 internalization. Our screen allows us to directly identify endocytic mutants using a high-throughput, phenotype-based approach to study endocytic mechanisms of specific cargo proteins, and to find mutants with altered rates of transit from the plasma membrane to early/late endosomes.

P2029**Board Number: B547****Defining the adaptor function of endocytic protein Syp1.**K. Hoban¹, A. Reider Apel², S. Chuartzman³, M. Schuldiner^{4,5}, B. Wendland¹;¹Department of Biology, Johns Hopkins University, Baltimore, MD, ²Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, ³Department of Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel, ⁴Department of Molecular Genetics, The Weizmann Institute of Science, Rehovot, Israel, ⁵Molecular Genetics, The Weizmann Institute of Science, Rehovot, Israel

Endocytosis is an important cellular process. One class of endocytic proteins is the adaptors, which bind to specific cargos, membranes, and other components of the endocytic machinery. One of the earliest-arriving proteins recruited to sites of clathrin-mediated endocytosis (CME) in yeast is Syp1, a member of the muniscin family of adaptors that includes multiple mammalian homologs.

Syp1 is known to bind Ede1, another early-arriving CME factor, and cargos such as Mid2, via its μ -homology domain (μ HD). However, the molecular mechanisms underlying Syp1's adaptor functions, including the sites on the μ HD necessary for its physical interactions with Ede1 and cargo proteins, as well as the sorting motifs that allow Syp1 to select specific cargos for internalization, have not been determined. Therefore, we aimed to locate the Ede1 binding site on the μ HD and ascertain the mechanisms of cargo recognition. Also, few proteins have been classified as Syp1 cargos; consequently, we sought to identify additional cargos of this adaptor.

Using a collection of alanine scanning μ HD mutants, we isolated an evolutionarily conserved region of the domain's surface that appears important for its physical interaction with Ede1. This construct, in which a seven residue-long sequence of the μ HD is mutated, shows reduced binding to Ede1 in assays such as affinity pull-downs. However, the mutant does bind Mid2, indicating that the mutant μ HD is folding properly and the decreased affinity for Ede1 is due to loss of side chains present in the WT domain.

We also confirm the identity of Mep3, an ammonium permease isolated in a high throughput microscopy screen, as a novel Syp1 cargo using fluorescence microscopy. Mep3-GFP internalization is inhibited in the absence of Syp1; the protein instead accumulates at the plasma membrane. Its endocytosis is also μ HD-dependent, as the introduction of truncated Syp1 lacking this domain fails to internalize Mep3. Similarly, truncation of a portion of the Mep3 cytoplasmic, C-terminal tail prevents its endocytosis in WT cells. This indicates that Mep3 is a novel Syp1 cargo recognized specifically by the μ HD, possibly via a specific sorting motif.

We are searching for this potentially novel endocytic motif involved in Syp1 cargo selection. We predict that a motif present in the cytoplasmic tail of Syp1 cargos such as Mid2 is important for cargo binding to the μ HD. Mutant Mid2 in which a small cytoplasmic segment is mutated to alanines exhibits diminished affinity for the μ HD in pull-down assays. We plan to use our collection of alanine scanning μ HD mutants

to identify the cargo binding site on this domain, complementing our studies to define the sorting motif within cargo proteins.

P2030

Board Number: B548

CALM is an adaptor for Gyating-clathrin coated membranes.

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We have identified highly dynamic clathrin-coated structures in the endosomal region of mammalian cells, and their rapid but highly localized movement, suggestive of clathrin-coated buds on membrane tubules, led to the term “Gyrating-“ or “G-clathrin” (1). Endocytic cargo such as transferrin can be visualized in G-clathrin downstream of the sorting endosome, and the structures have been implicated in rapid recycling of transferrin, β_1 -integrin and c-met receptor. While the tetrameric TGN and plasma membrane adaptors AP-1 and AP-2 are virtually undetectable in G-clathrin the single chain adaptors GGA1 or GGA3, expressed as fluorescent protein fusions, are almost completely colocalized with G-clathrin. Here we report that another single chain adaptor, GFP-tagged CALM (Clathrin assembly lymphoid myeloid leukemia protein) is also present in G-clathrin. Importantly, endogenous CALM can also be detected in G-clathrin structures followed through fixation. CALM contains an unstructured assembly domain which binds clathrin, and an N-terminal phosphoinositide-binding ANTH domain which has also recently been shown to bind SNARE proteins (2). Consistent with this, we could readily detect GFP-tagged forms of VAMP3, VAMP4 and VAMP8 in G-clathrin. However, SNARE binding is not critical for G-clathrin formation, as G-clathrin persisted in cells over-expressing either CALM mutants lacking SNARE binding or a CALM construct lacking the entire ANTH domain, and all were incorporated into G-clathrin structures. In contrast, expression of the CALM ANTH domain alone was cytosolic (3). G-clathrin also was unaffected by siRNA-mediated knockdown of CALM. However, this treatment abolished appearance of VAMP3 in G-clathrin. As endocytosis of VAMP3 is blocked under these conditions, as previously reported (2), these observations indicate that G-clathrin functions primarily in VAMP3 recycling rather than in biosynthetic transport, and that CALM is not required for G-clathrin formation. Finally, in cells expressing tagged forms of both CALM and GGA1, their presence in G-clathrin appeared mutually exclusive. While structures with GGA1 are primarily Arf6-dependent and are resistant to BFA, those with CALM show substantial sensitivity to BFA. This suggests that G-clathrin, until now defined only by its mobility and clathrin coat, likely exists in structurally and functionally distinct forms in the endosomal regions of cells. Further, our results suggest that G-clathrin is a significant trafficking compartment for other potential CALM cargos.

1- Zhao et al., *Traffic* **9**:2253 (2008)

2- Miller et al., *Cell* **147**:1118 (2011)

3- Tebar et al., *Mol Biol Cell* **10**:2687 (1999)

P2031**Board Number: B549****The use of phospholipase A2 Inhibitors to investigate the localization and trafficking of amyloid precursor protein.**S.K. Boyle¹, N. Belko¹, E.B. Cluett¹;¹Biology, Ithaca College, Ithaca, NY

Evidence from our lab has implicated phospholipase A2 activity in the intracellular transport of LDL-derived free cholesterol in the endomembrane system. The effects of phospholipase inhibitors on the localization of proteins associated with endocytic compartments implicated the early endosome and the Endocytic Recycling Compartment (ERC) in the transport of cholesterol. siRNA knockdown of rab5 isoforms revealed differential effects on the distribution of cholesterol in both control and ONO-treated cells. Using isoform-specific antibodies and immunofluorescence microscopy, treatment with ONO altered the localization of rab5A and rab5B but not rab5C. The ability to alter the distributions of cholesterol and marker proteins associated with specific intracellular compartments suggest that phospholipase A2 inhibitors may be useful in investigating the trafficking and processing of amyloid precursor protein (APP), a normal cellular protein that undergoes complex proteolytic processing. Misprocessing of this protein is associated with Alzheimer's Disease. However, it is still not clear where the different processing events occur in the cell or what causes the misprocessing. Cholesterol is implicated in the onset of AD, and evidence suggests that the amount of cholesterol in the membrane may play a role in the misprocessing of APP. An emerging model suggests that one set of processing steps occur on the plasma membrane, but alternative processing, which can lead to misprocessing, may occur in an intracellular compartment. In CHO cells that have been stably transfected with APP, the protein is found in several different intracellular compartments, which is consistent with existing evidence. Treatment of these cells with ONO significantly changes the distribution of APP. However, APP does not appear to be appreciably associated with cholesterol in the ERC. Knockdowns of rab5 isoforms or rab11 change the distribution of APP suggesting that the protein traffics through the endocytic pathway. Experiments are now underway using immunoblotting and sandwich ELISAs to investigate the processing of the protein in the presence and absence of ONO.

P2032**Board Number: B550****cholesterol trafficking between the plasma membrane and the endocytic recycling compartment.**S. Mao¹, D.B. Iaea^{2,3}, F.R. Maxfield²;¹biochemistry, Cornell University Weill Cornell Medical College, New York, NY, ²Cornell University Weill Cornell Medical College, New York, NY, ³Tri-Institutional Program in Chemical Biology, Weill Cornell Medical College, New York, NY

Cholesterol is an essential constituent of membranes in mammalian cells. The plasma membrane (PM) is the largest cholesterol pool in the cell and contains ~60% of total cellular cholesterol. Cholesterol is also highly enriched in the endocytic recycling compartment (ERC). The abundance and distribution of intracellular cholesterol within membranes are tightly controlled by complex and poorly characterized homeostatic mechanisms. To analyze cholesterol transport between organelles, a fluorescent cholesterol analog dehydroergosterol (DHE) was used. We used fluorescence recovery after photobleaching (FRAP) to measure cholesterol delivery to ERC. Within 30 min after photobleaching, the fluorescence in ERC recovered about 90%. Recovery of DHE fluorescence in the ERC can be described by a single first order rate constant. The recovery $t_{1/2}$ into the ERC is 14.5 ± 1.5 min in U2OS cells. We developed a novel efflux method, in which a large exogenous cholesterol pool from red blood cells (10^9 per ml) rapidly exchanges with DHE at PM. This new method allows for accurate measurement of DHE efflux that can be modeled as a single exponential to characterize sterol transport rates from the ERC to the PM. DHE in the ERC effluxed completely out of cells with a $t_{1/2}$ of 14.9 ± 0.9 min in U2OS cells. Using these experimental improvements in the measurements of DHE trafficking, we accurately measured the effect of intracellular cholesterol level, the role of cholesterol transporters, and the energy-dependent and -independent transport processes in sterol trafficking. High intracellular cholesterol levels increase cholesterol the transport rate constant between the ERC and the PM, while mildly reduced intracellular cholesterol levels do not statistically change the rate constants for cholesterol transport into and out of the ERC. The increased amount of a cholesterol transporter (STARD4) significantly enhances the rate of cholesterol transport between ERC and PM. Energy depletion slightly reduces the rate of cholesterol transport between the ERC and the PM. These results suggest that while there is some contribution from vesicular transport, nonvesicular transport plays the major role in sterol transport between the ERC and the plasma membrane.

P2033

Board Number: B551

CFTR transcytosis in human respiratory epithelia: a constitutive rescue mechanism of basolaterally missorted channel from the biosynthetic and recycling pathways.

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Cystic Fibrosis (CF) is caused by mutations in the CF transmembrane conductance regulator (CFTR), a chloride channel in the apical (Ap) plasma membrane (PM) of secretory epithelia. Considering that CFTR density at the Ap PM correlates with the CF disease severity, understanding the cellular/molecular mechanisms that determine the channel delivery to and removal from the Ap PM has fundamental importance. The mechanism responsible for CFTR polarized expression, however, remains poorly defined. Using morphological and biochemical assays, here we show that CFTR undergoes constitutive transcytosis (basolateral-to-apical) in differentiated, filter grown immortalized (CFBE) and conditionally

reprogrammed primary (HBE) human bronchial epithelial cells expressing CFTR. Transcytotic CFTR partly originates from basolaterally delivered channel from a) non-selective Ap and basolateral (Bl) delivery of secretory vesicles (cca. 30%) and b) from reversed or Ap-to-Bl transcytosis (cca. 70%) as a consequence of the limited fidelity of Ap recycling internalized CFTR. A similar trafficking rescue mechanism may be involved in mutations leading to small C-terminal truncations that have been implicated in compromising CFTR polarized expression by disrupting the interaction between the C-terminal PDZ (Postsynaptic density/Disc large/ZO-1)-binding motif of CFTR and NHERF-1, a PDZ-domain containing scaffolding protein. Truncation of the PDZ-binding motif increased internalization and decreased endocytic recycling of the CFTR- $\Delta 6$ mutant without discernible effect on Ap expression and stability in CFBE cells contrary to previous reports. The preserved Ap expression of CFTR- $\Delta 6$ could be explained by its increased transcytosis rate, similarly to that of the wild-type CFTR upon NHERF-1 knockdown and accounts for the lack of CF clinical phenotype in individuals carrying C-terminal truncations. Using morphological, genetic and biochemical tools some unique characteristics of CFTR transcytosis were revealed. Internalized CFTR delivery to contralateral PMs avoids both Ap and Bl recycling endocytic compartments contrary to Transferrin and IgA receptors, but likely involves EEA1 positive early endosomes. On the other hand, microtubule disruption interferes with the transcytosis of multiple cargoes, including CFTR, without interfering with its internalization and recycling. In summary, we have uncovered some of the cellular-molecular mechanisms that contribute to CFTR transcytosis and plays a role in the channel polarized expression in airway epithelial cells by redirecting basolaterally missorted molecules to the apical PM.

P2034

Board Number: B552

Shear Stress Dependent Regulation of Apical Endocytosis in Renal Proximal Tubule Epithelia.

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The kidney has an extraordinary ability to maintain stable fractional solute and fluid reabsorption over a wide range of glomerular filtration rates (GFRs). Internalization of filtered low molecular weight proteins, vitamins, hormones, and other small molecules is mediated by the proximal tubule (PT) multiligand receptors megalin and cubilin. Changes in GFR and the accompanying fluid shear stress (FSS) modulate acute changes in PT ion transport thought to be mediated by microvillar bending. We recently discovered that FSS also modulates apical endocytosis in PT cells via a mechanosensitive pathway regulated by primary cilia. FSS caused an acute and transient increase in intracellular calcium in cells that preceded the endocytic response. Neither the FSS-stimulated increase in intracellular calcium or apical endocytosis was observed in deciliated cells. Addition of ATP to the medium rescued both responses, suggesting the involvement of purinergic receptors in the signaling cascade. Consistent with this, inclusion of the ATP hydrolyzing enzyme apyrase or the pan-purinergic receptor inhibitor suramin in the perfusion medium blocked the FSS-stimulated endocytic response. Both basal and FSS-stimulated

endocytosis were inhibited by perturbants of clathrin and dynamin function. Additionally, treatment of PT cells with small molecule inhibitors of cdc42 or siRNA mediated knockdown of cdc42 ablated the FSS-stimulated increase in endocytosis. Similarly knockdown of N-WASP or inhibition of Arp2/3 complex-mediated nucleation of branched actin filaments prevented the FSS stimulated endocytic response. Our data suggest that exposure of PT cells to FSS enhances apical clathrin-mediated endocytosis via an actin-dependent pathway that is modulated by activation of Cdc42, N-WASP, and Arp2/3. Studies are underway to determine how the FSS-stimulated increases in intracellular calcium are transduced to increases in endocytosis.

P2035

Board Number: B553

Apical and Basolateral Recycling in Epithelial Cells.

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Epithelial cells mediate vectorial transport of solutes and nutrients, by accurately sorting transporters and receptors to the apical and basolateral plasma membrane (PM) domains. Whereas some sorting occurs in the biosynthetic route, the major sorting of fast recycling receptors, such as Megalin and Transferrin receptor (TfR), occurs in the recycling route. Megalin is an apical recycling receptor responsible for the absorption of ultrafiltrate proteins in the kidney proximal tubule and its mutation in several human genetic syndromes causes proteinuria. TfR is the best-studied basolateral recycling receptor, which mediates iron uptake from the blood. Whereas the basolateral recycling pathway has been extensively studied using several endocytic receptors (i.e. TfR, LDLR, EGFR, etc), the apical recycling pathway is poorly understood. Here we show that apically internalized Megalin recycles successively through WGA-positive apical sorting endosomes, TfR-positive common recycling endosomes (CRE) and Rab11a-positive apical recycling endosomes (ARE) with half-times of 5, 12 and 21 minutes, respectively. Apically internalized Megalin mixes with basolaterally internalized TfR at CRE, suggesting that this is the compartment where sorting of these recycling receptors occurs. Very little is known on the mechanisms that mediate sorting and recycling of Megalin from CRE to the apical PM. Previous work has identified NPXY-like apical sorting signals (Takeda et al., *AJP*, 2003; Marzolo et al., *Traffic*, 2003) and our preliminary results suggest that clathrin is involved. In contrast, basolaterally internalized TfR is known to be sorted at CRE, by the clathrin adaptor AP-1B and clathrin, into a recycling route to the basolateral membrane. In certain epithelia that constitutively lack AP-1B, such as Retinal Pigment Epithelium, and in AP-1B knocked-down MDCK cells, TfR is relocated to the apical surface through a novel transcytotic pathway that involves its apical sorting at CRE by an N-glycan sorting signal and the sorting lectin galectin-4 (Perez Bay et al., *JCS*, 2014) and its transport from CRE to ARE and the apical surface by the kinesin KIF16B, non-centrosomal microtubules and the small GTPase rab11a (Perez Bay et al, *EMBO J*, 2013). In summary, our results describe the complete endosomal itinerary of apically

recycling megalin, provide the first evidence for a role of clathrin in apical sorting in mammalian cells and identify a novel transcytotic pathway for basolateral recycling receptors in AP-1B deficient epithelia.

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P2036

Board Number: B554

The Interaction of the Human Papillomavirus type 18 E6 Oncoprotein with Sorting Nexin 27 Modulates Retromer Dependent Cargo Recycling Function.

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Human Papilloma viruses are the causal agents of a large number of human malignancies, chief among which is cervical cancer. The continued over expression of the viral oncoproteins E6 and E7 is required for the initiation and maintenance of the neoplasm. Cancer causing HPV types are termed as high risk and a defining feature of the E6 oncoprotein from these types is the presence of a C-terminal PDZ binding motif. This allows the E6 protein to bind to and target various cellular PDZ domain containing proteins for degradation. Recent proteomic studies by Rozenblatt-Rozen et al (2012) and Belotti et al (2013) show that E6 may potentially bind to SNX27, which is a component of the endosomal transport system. Sorting Nexin 27 is unusual in that it is the only sorting nexin known to have a PDZ domain. This PDZ domain is known to facilitate the interaction of SNX27 with various PBM containing cargoes, thus directing their transport through the endosomal system. In this study, we show that SNX27 is a bonafide binding partner of HPV18E6 and this interaction is PDZ dependent. Furthermore, this interaction is conserved across various high risk cancer causing HPV types. We also show that E6 alters the sub-cellular distribution of SNX27 in HeLa cells and disrupts the SNX27-retromer link in a PDZ dependent manner. Analysis of known SNX27 cargoes such as the glucose transporter GLUT1 shows a perturbation in cargo retrieval and transport in the presence of E6. We show that the loss of E6 reduces surface GLUT1 levels in HeLa cells and this loss is reflected in increased lysosomal accumulation as seen in endosomal fractionation studies. We also demonstrate that the loss of E6 re-establishes the interaction between the retromer and GLUT1 in HeLa cells; further supporting the hypothesis that E6 is involved in the perturbation of the SNX27-retromer recycling pathway. Thus, these studies demonstrate a novel function for high risk HPV oncoproteins in the regulation of endosomal sorting pathways.

P2037**Board Number: B555****Defining Cytosolic Components That Regulate Endosomal Membrane Protein Sorting.**M.B. Gireud^{1,2}, S. Reyes^{1,2}, N. Sirisaengtaksin^{1,2}, S. Tsunoda³, A.J. Bean^{1,2,4};¹Neurobiology and Anatomy, University of Texas Health Science Center at Houston, Houston, TX,²University of Texas Graduate School of Biomedical Sciences at Houston, Houston, TX, ³BiomedicalSciences, Colorado State University, Fort Collins, CO, ⁴Pediatrics, M.D. Anderson Cancer Center, Houston, TX

Endocytosis is required for the uptake of essential nutrients from the extracellular environment and to limit the signaling by molecules (e.g. ion channels, receptors) present on the plasma membrane. The endocytic pathway is separated into stages based on cargo movement and identification of morphologically defined compartments. After internalization and transport from early to late endosomes, proteins destined for degradation in the lysosome are internalized into the lumen of late endosomes via membrane invagination and vesicle fission. The resulting organelle, the multivesicular body (MVB), is characterized by a limiting membrane and the presence of internal vesicles. Proteins that are sorted into internal vesicles are subject to degradation while those that remain on the limiting membrane are recycled. To facilitate sorting of proteins into the internal vesicles of the MVB, ubiquitin is added to the membrane protein. Ubiquitin allows for interaction with components of the sorting machinery, a group of cytosolic multi-protein complexes, the endosomal sorting complexes required for transport (ESCRTs), that play a role in protein sorting and internal vesicle formation.

To examine the mechanisms underlying endosomal sorting, we have established a cell-free based assay that measures both MVB formation (ultrastructure) and the sorting of a cargo protein into the endosomal lumen. The sorting event is dependent on cytosolic components, ATP, time and temperature. Since this sorting event is dependent on soluble proteins, we examined the requirements and regulation of endosomal sorting by proteins present in the cytosol. We have undertaken a biochemical examination of cytosolic components by taking advantage of yeast and fly genetics, to understand the regulation of endosomal sorting and MVB biogenesis.

By following the movement of a prototypical membrane protein, the epidermal growth factor receptor (EGFR), we have determined that cytosol obtained from *Drosophila Melanogaster* fly strains and *Saccharomyces cerevisiae* yeast strains are sufficient to support the endosomal sorting of the EGFR with reasonable efficiency (20-40%). Further, cytosol derived from yeast strains deleted of ESCRT-0-III components decreased EGFR sorting into endosomes. The addition of recombinant mammalian ESCRT-0 homologs rescues the sorting defect caused by the absence of these yeast proteins. We have also examined other non-ESCRT cytosolic proteins that regulate the MVB sorting process. Understanding the molecular details of this critical endosomal sorting step will allow a better appreciation of the molecular pathways that ultimately may affect membrane protein signaling.

P2038**Board Number: B556****Exploring retromer de-regulation in Parkinson's disease.**

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Retromer is a protein assembly that plays a central role in orchestrating export of trans-membrane cargo proteins from endosomes into recycling pathways destined for the Golgi apparatus and the plasma membrane [1]. Recently, a specific mutation in the retromer component VPS35, VPS35 (p.D620N), has linked retromer dysfunction to familial autosomal dominant and sporadic Parkinson's disease [2, 3].

Here we established that in cells expressing VPS35 (D620N) there is a perturbation in endosome-to-Golgi transport but not endosome-to-plasma membrane recycling, which we confirm and functionally rescue in Parkinson's disease patient cells harboring the VPS35 (p.D620N) mutation [4]. Through comparative stable isotope labeling by amino acids in cell culture (SILAC)-based analysis of wild-type VPS35 versus the VPS35 (p.D620N) mutant interactomes, we establish that the major defect of the D620N mutation lies in the association to the actin nucleating Wiskott-Aldrich syndrome and SCAR homologue (WASH) complex [4,5]. Consistent with this we quantify, using isothermal calorimetry that the affinity of VPS35 (p.D620N) for the WASH complex component FAM21 is decreased by 2.2 +/- 0.5 fold compared to wild-type [4].

In addition, we confirm the interaction between retromer and DNAJC13 [6], a mutation in which, DNAJC13 (N855S), is also linked with Parkinson's disease [7]. Together with evidence that retromer associates with LRRK2 [8] these data highlight retromer-mediated endosome sorting as a new pathway for studying the pathophysiology of Parkinson's disease. Ongoing studies seek to utilize iPSCs and mouse models to further elucidate the role of retromer in dopaminergic neuron function and viability.

[1] Cullen PJ, Korswagen HC. *Nat Cell Biol* 14, 29-37 (2012). [2] Vilarino-Guell et al. *Am J Hum Genet* 89, 162-167 (2011) [3] Zimprich et al. *Am J Hum Genet* 89,168-175 (2011). [4] McGough et al. *Curr Biol* 24, 1670-1676 (2014). [5] Zavodszky et al. *Nat Commun* 5, 3828 (2014). [6] Freeman et al. *J Cell Sci* 127, 2053-2070 (2014). [7] Vilarino-Guell et al. *Hum Mol Genet* 23, 1794-1801 (2014). [8] MacLeod et al. *Neuron* 77, 425-439 (2013).

P2039**Board Number: B557****Numb Diverts Notch to Late Endosomes to Antagonize Notch Membrane Recycling in Drosophila Sensory Organs.**S. Johnson¹, F.J. Roegiers²;¹Cell and Molecular Biology, University of Pennsylvania, Philadelphia, PA, ²Fox Chase Cancer Center, Philadelphia, PA

Notch signaling is a well conserved signaling pathway that controls a wide variety of developmental functions including cell fate determination and patterning. Errors in the regulation Notch signaling have been implicated in forms of colon cancer and leukemia, as well as other leukodystrophies including CADASIL syndrome. To elucidate Notch signaling regulation, we took advantage of sensory organ differentiation in Drosophila, a process that requires regulation of both Notch activation and inhibition. Sensory organs of Drosophila arise from a pair of cells, one of which must activate Notch signaling, while the other must inhibit signaling. Blockage of Notch signaling in one of the cells requires the presence of Numb, a membrane-associated protein whose mechanism of Notch inhibition is currently unknown. We are working to show that Numb functions through preventing the recycling of the Notch receptor back to the plasma membrane. We show that Numb promotes targeting of full-length Notch to late endosomes. Conversely, Notch levels in early endosomes are equivalent between the Numb positive and negative cells, supporting a model where Numb functions in Notch trafficking at a post-internalization step. Furthermore, we find that Numb itself may localize to recycling endosomes. To evaluate Notch recycling directly, we employed a novel assay that allows for marking of recycled vs. internalized Notch. Using this technique we found that Numb suppresses Notch receptor recycling back to the plasma membrane. Our work will provide new insight into the inhibition of Notch via Numb, which may allow for the creation of new drugs which can function similarly to Numb to regulate Notch activity.

P2040**Board Number: B558****Clathrin Independent Endosomal Trafficking is required for T Cell Conjugate Formation and Activation.**D. Johnson¹, J.M. Wilson², J.G. Donaldson³;¹Univ Arizona, Tucson, AZ, ²Univ Arizona Coll Med, Tucson, AZ, ³Cell Biology and Physiology Center, NHLBI/NIH, Bethesda, MD

The clathrin independent endosomal system is important for cellular homeostasis and specialized modifications of the plasma membrane. We characterized clathrin independent endocytosis (CIE) in the human Jurkat T cell line and compared it other cells. We found that major histocompatibility complex I

(MHCI) was endocytosed separately from the clathrin-mediated endocytic marker the transferrin receptor. Subsequently, MHCI passed through EEA1-positive endosomes, prior to trafficking to LAMP-1 positive vesicles for degradation or to endosomes for recycling back to the plasma membrane. In contrast, another CIE cargo protein, CD98, avoided residence in EEA1 positive endosomes after internalization and was rapidly recycled back to the cell surface. Thus CIE in Jurkat cells is similar to what is observed in other human cell lines.

Two G proteins, Arf6 and Rab22, have been shown to regulate endosomal trafficking and recycling of CIE cargo proteins. They are also required for rearrangement of the cell surface during events like cell spreading. We found that wild type Arf6 co-localized with CIE cargo and Rab22 co-localized with internalized MHCI in resting T cells. To test whether Arf6 or Rab22 activities were required for T cell activation we incubated Jurkat cells with activated antigen presenting cells (APC). Expression of the dominant negative mutants of Arf6 or Rab22 decreased T cell engagement with the APC and reduced actin polarization at the site of the immunological synapse. In addition, there is reduced T cell signaling as indicated by decreased phosphorylated tyrosine at the synapse of cells expressing mutant Arf6. Arf6 and Rab22 dominant negative mutants were also inhibited in their ability to spread when plated on antibody-coated coverslips that cause activation.

Our results confirm that CIE and subsequent endosomal trafficking is a highly conserved process. The clathrin independent endosomal system is used by T cells to redistribute and polarize membranes to the site of activation. Our findings suggest a requirement for Arf6 and Rab22 in mediating membrane trafficking and signaling events at the immunological synapse. Our future goal is to understand how CIE and endosomal trafficking affects T cell signaling and function.

P2041

Board Number: B559

Macronutrient stress modulates antigen trafficking and MHC class II presentation by altering HSC70.

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B lymphocytes exploit macroautophagy to capture cytoplasmic and nuclear proteins within autophagosomes. Autophagosome fusion with lysosomes and endosomes facilitates content proteolysis, with the resulting peptides selectively binding MHC class II molecules which are displayed for recognition by T lymphocytes. Nutrient stress amplified this pathway, favoring increased class II presentation of cytoplasmic antigens targeted to autophagosomes. By contrast, nutrient stress diminished class II presentation of membrane antigens including the B cell receptor (BCR) and cytoplasmic proteins that utilize the chaperone-mediated autophagy (CMA) pathway. While intracellular protease activity increased with nutrient stress, endocytic trafficking and proteolytic turnover of the BCR was impaired. Addition of high molecular mass proteins restored endocytosis and antigen presentation, evidence of tightly regulated membrane trafficking dependent on macronutrient status. Altering the

abundance of the cytosolic chaperone HSC70 was sufficient to overcome the inhibitory effects of nutritional stress on BCR trafficking and antigen presentation. Together, these results reveal a key role for macronutrient sensing in regulating immune recognition and the importance of HSC70 in modulating distinct membrane trafficking pathways during cellular stress.

P2042

Board Number: B560

Alterations in Trafficking and Degradation of FGFR4, a commonly mutated protein in High-Risk Neuroblastoma Cases.

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Overall survival rates for children diagnosed with high-risk neuroblastoma remain low despite current treatment regimens. Resistance of these tumors to conventional therapies is a major problem and the molecular mechanisms that underlie resistance remain unclear. Growth factor receptors and their signaling cascades have been suggested to cause tumorigenesis and/or metastasis. Therapies targeting these receptors are often effective anti-cancer agents. The fibroblast growth factor receptor 4 (FGFR4) signaling cascade promotes cell division and differentiation. We have observed that expression of a polymorphic FGFR4 correlates with increased mortality in neuroblastoma patients. This polymorphism generates a glycine-to-arginine change in codon 388. It has been linked to aggressive phenotypes in various other cancers and is associated with increased signaling. FGFR4 signaling can be regulated by the endocytic pathway such that the duration and amplitude of receptor signal can be altered by receptor trafficking. Upon ligand binding, FGFR4 is internalized and moves through a series of endosomal compartments prior to being degraded in the lysosome. We hypothesize that the polymorphism (Arg388) interferes with trafficking of this receptor through the endocytic pathway and that sustained signaling from non-degraded receptors promotes neuroblastoma growth. Given the apparent importance of this FGFR4 mutation in neuroblastoma tumorigenesis, and the role that endocytic trafficking can play in receptor signaling kinetics, we explored whether this polymorphism displayed altered trafficking that might underlie enhanced signaling.

We examined (wt) Gly388 and (mutant) Arg388 FGFR4 degradation in SY5Y cells following ligand (bFGF) stimulation. We observed that FGFR4 Arg388 receptors are degraded at a slower rate than wild-type FGFR4 receptors. To understand where in the endocytic pathway the mutant FGFR4 is delayed, we compared the movement of FGFR4s through various steps in the endocytic pathway. For example, we used a cell-free assay that measures movement of membrane proteins into internal vesicles of late endosomes to examine whether wt or mutant FGFR4 are differentially sorted at the late endosome.

Proteins sorted into internal vesicles within late endosome/MVBs are degraded upon fusion with the lysosome and decreased MVB sorting could therefore account for decreased degradation. However, no significant differences in MVB sorting were observed between wild-type FGFR4 and FGFR4 Arg388. Analysis of other endocytic steps whose inhibition could account for decreased FGFR4 Arg388 degradation (e.g. internalization from the plasma membrane, recycling to the plasma membrane, and movement from early endosomes) as well as effects on downstream signaling are ongoing.

P2043

Board Number: B561

Divide and Polarize: The Role of Cytokinesis and Endocytic Transport during Epithelial Cell Polarization.

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Epithelial cells are structurally and functionally polarized to transport specific molecules while maintaining a trans-epithelial barrier. This cellular asymmetry is essential for the proper functioning of epithelial tissues and depends on polarized endocytic transport routes. Additionally, epithelial cells coordinate their polarization with neighboring cells to form an apical lumen, a key step in the establishment of renal and gut architecture, and thereby function. Recent work from several laboratories including ours identified Rab11 and its binding protein FIP5 as major components that regulate apical endosome transport during apical lumen formation. We demonstrated that Rab11/FIP5-containing endosomes (FIP5-endosomes) mediate the formation of apical lumen by targeted delivery of apical lumen proteins, and that FIP5 functions by interacting with sorting nexin 18 and Kinesin-2. It was also shown that targeting of FIP5-endosomes to the apical membrane initiation site (AMIS) is a key step during the initiation and expansion of the apical lumen during formation of MDCK epithelial cysts in 3D tissue culture system. Despite recent advances in our understanding of the mechanisms mediating lumen formation, many questions remains unanswered. How are FIP5-endosomes targeted during apical lumen formation? How do cells establish the site of single apical lumen? In this study we focused on the identification of the machinery that mediates AMIS formation and FIP5-endosomes targeting during apical lumen formation. We identified a new FIP5-interacting protein, cingulin, which is a tight junction protein that localizes to the AMIS. Thus, cingulin is perfectly suited to serve as a tether to mediate FIP5-endosome targeting during apical lumen formation. Importantly, our data demonstrate that AMIS forms around the midbody of the mitotic epithelial cells during late telophase. Finally, we demonstrate that AMIS formation is initiated by Rac1-dependent burst of actin polymerization at the midbody. Based on all these studies we propose a novel hypothesis that AMIS formation around the midbody and FIP5/cingulin-dependent endosome targeting to the midbody during late telophase are the first “symmetry breaking” events leading to the initiation of apical lumen during epithelial morphogenesis.

P2044**Board Number: B562****Novel mechanistic insights into anthrax toxin endocytosis.**S. Friebe¹, L. Abrami¹, F. Van Der Goot¹;¹EPFL, Lausanne, Switzerland

Anthrax is a disease largely caused by the exotoxins of *Bacillus anthracis*. Entry into cells is mediated by one of the two known anthrax toxin receptors. The main receptor is Capillary Morphogenesis Gene 2 (CMG2) also known as ANTXR2, a single pass type I transmembrane protein of unknown physiological function. How these toxins act on cells is well studied, yet the exact initial steps of toxin entry are still poorly understood. We therefore aimed to better understand this complex and fascinating process and to shed light on the physiological role of the main anthrax toxin receptor. For this we identified new potential partner proteins of CMG2 by using several Yeast Two Hybrid screens:

Currently our work is focusing on a kinase and an E3 ubiquitin ligase as well as a protein identified from a genome wide screen for anthrax susceptibility, ARAP3. ARAP3 is a PI3K effector protein and a RhoGAP implicated in integrin regulation and growth factor receptor trafficking. Lastly, the downstream effector of ARAP3, RhoA is a protein known to mediate actin reorganization upon extracellular stimuli.

We could show that all proteins interact with CMG2 by co-immunoprecipitation and have started to pinpoint the interacting domains on the proteins by biochemical and microscopical methods. We also show that interaction between the proteins is constant and not ligand-triggered for all four proteins. To understand the functional implications of these interactions we studied the different steps of anthrax toxin endocytosis. Knockdown of all four proteins did not have an effect on binding of the toxin, yet the effect on later steps of the process was striking. In the absence of the kinase and the E3 ligase, endocytosis of the receptor-toxin complex was almost completely inhibited. This might be due to a lack of phosphorylation and ubiquitination of the receptor after toxin binding in the absence of these enzymes. Knockdown of ARAP3 strongly delayed endocytosis and knockdown of RhoA led to a defect of heptamer formation, a prerequisite for efficient endocytosis. To ensure that these proteins act specifically on CMG2, we looked at entry of Diphtheria Toxin, which also enters cells via clathrin-mediated endocytosis. So far it seems that the proteins are specific for endocytosis of anthrax and are not general regulators of clathrin-mediated endocytosis. By gaining important insights into the endocytosis of anthrax toxin with the help of this small-scale protein network, we also hope to broaden our knowledge about the physiological behavior of its receptor.

P2045**Board Number: B563****Ack1 Overexpression Results in the Interruption of Several Endocytic Pathways and Vesicular Trafficking.**C. Ye¹, L. Guan¹, T. Lin¹, V.E. Prieto-Echagüe², D.A. Brown³, W.T. Miller⁴, A.L. Gucwa¹;¹Biomedical Sciences, Long Island University At Post, Brookville, NY, ²Molecular Human Genetics, Institut Pasteur de Montevideo, Montevideo, Uruguay, ³Biochemistry and Cell Biology, Stony Brook University, Stony Brook, NY, ⁴Physiology and Biophysics, Stony Brook University, Stony Brook, NY

Ack1 is a direct effector of the Rho GTPase member Cdc42, and is amplified and overexpressed in several types of cancers. Its strong implications in tumor progression and metastasis make it of great interest. However, until recently, little was known about this non-receptor tyrosine kinase (NRTK) and its regulation. The NRTK is known to play an integral role in clathrin-dependent internalization and the down-regulation of activated EGFR. We were interested to see whether Ack1 also plays a role in the internalization and trafficking of ErbB2, a member of the ErbB family of growth factor receptors. Treatment of cells in culture with the ansamycin antibiotic, geldanamycin (GA), results in the internalization and degradation of ErbB2 in a clathrin-independent manner. The overexpression of Ack1 disrupted the down-regulation of ErbB2 in GA-treated cells. Cholera toxin uptake, which occurs via lipid raft-dependent mechanisms, was also blocked by its overexpression. We did not find any effect of Ack1 on bulk fluid uptake, as the internalization of high-molecular weight dextran was unchanged. Additionally, we found this effect was not limited to endocytosis; Ack1 overexpression induced the disruption and tubularization of the Golgi apparatus, similar to that seen in Brefeldin A-treated cells. To further investigate the role of Ack1 on the trans-Golgi network, we studied its effect on mannose 6-phosphate receptor (MPR). This receptor binds newly synthesized lysosomal hydrolases in the trans-Golgi network (TGN) and delivers them to pre-lysosomal compartments. A GFP-tagged form of cation-independent MPR (CI-MPR) was sequestered at the plasma membrane when co-expressed with Ack1, suggesting Ack1 may also alter retrograde transport. Taken together, our data suggests the overexpression of Ack1 plays a global and more significant role in endocytosis and intracellular trafficking than previously thought.

P2046**Board Number: B564****FAM21 regulates recycling of SNX27-retromer cargoes to the plasma membrane.**S. Lee¹, J. Chang¹, C.D. Blackstone¹;¹NINDS, National Institutes of Health, Bethesda, MD

The endosomal network controls numerous cellular functions such as signaling, nutrient uptake, and development through balanced trafficking of diverse plasma membrane proteins. After receiving membrane protein cargoes, endosomes sort them into lysosomes for degradation, or else to the trans-Golgi network or plasma membrane for recycling. These pathways are elaborately controlled by the

retromer complex, a multi-subunit complex associated with the cytosolic face of endosomes, along with its interacting proteins. Recently, the retromer complex in association with SNX27, a PDZ-domain-containing SNX protein, has been identified as a major endosomal hub to regulate endosome-to-plasma membrane recycling. Even though the Wiskott-Aldrich syndrome protein and SCAR homolog (WASH) complex, an activator of Arp2/3-mediated actin nucleation in endosomes, has been identified to interact with the SNX27-retromer complex, a specific role for the WASH complex in the recycling pathway has not been elucidated. Here, we have identified the FAM21 subunit of the WASH complex as a direct interacting partner of SNX27. A FERM domain of SNX27 binds to α -helical domains at the N-terminus of FAM21, which are distinct from the binding sites for the VPS35 subunit of the retromer complex. We have also demonstrated that the interaction is required for proper recycling of SNX27-retromer cargoes by distributing SNX27 within an endosomal subdomain to aid export of cargoes to their destination. Thus, we suggest that the WASH complex regulates endosome-to-plasma membrane recycling in collaboration with the SNX27-retromer complex.

P2047

Board Number: B565

A CLATHRIN AND DYNAMIN-INDEPENDENT PATHWAY OF SYNAPTIC VESICLE RECYCLING MEDIATED BY BULK ENDOCYTOSIS.

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The massive exocytosis of synaptic vesicles (SVs) elicited by a potent nerve terminal stimulation is rapidly compensated by bulk endocytosis of SV membranes leading to large endocytic vacuoles. Subsequently, these vacuoles ("bulk" endosomes) disappear in parallel with the reappearance of new SVs. We have capitalized on synapses of dynamin 1 and 3 double knock-out (DKO) neurons, where clathrin-mediated endocytosis is dramatically impaired, to gain insight into the poorly understood mechanisms underlying this process. Massive formation of bulk endosomes was not impaired in these neurons. Conversion of bulk endosomes into SVs during recovery was delayed, but not blocked, even after further clathrin knock-down, showing that this process is independent of clathrin-mediated budding, consistent with the absence of clathrin coats on bulk endosomes. These findings support the existence of a pathway for SV reformation that bypasses the requirement for clathrin and dynamin and demonstrates the plasticity of the SV recycling process.

Establishment and Maintenance of Polarity 2

P2048

Board Number: B567

Molecular insights into zipcode activated cytoplasmic mRNA transport.

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Myo4p is one of five distinct myosins of yeast, which is dedicated to cytoplasmic transport of two types of cargos, zipcoded mRNPs and tubular endoplasmic reticulum (tER). Neither cargo binds directly to Myo4p. Instead, She3p serves as 'adaptor' that contains three binding modules, one for Myo4p and one each for zipcoded mRNA and tER. The assembly of a transport-competent motor complex is poorly understood. Here, we report that [Myo4p•She3p] forms a stable 1:2 heterotrimer in solution. In the [Myo4p•She3p] crystal structure, Myo4p's C-terminal domain (CTD) assumes a lobster claw-shaped form, the minor prong of which adheres to a pseudo-coiled coil region of She3p. Because the [Myo4p•She3p] heterotrimer contains only one myosin molecule, it is not transport-competent. By stepwise reconstitution, we found a single molecule of synthetic oligonucleotide (representing mRNA zipcode element) bound to a single tetramer of zipcode binding protein She2p to be sufficient for [Myo4p•She3p] dimerization. Hence, cargo initiates cross-linking of two [Myo4p•She3p] heterotrimers to an ensemble that contains two myosin molecules obligatory for movement. An additional crystal structure comprising an overlapping upstream portion of She3p showed continuation of the pseudo-coiled coil structure and revealed another highly conserved surface region. We suggest this region as candidate binding site for a yet unidentified tER ligand. We propose a model, whereby zipcoded mRNA and/or tER ligands couple two [Myo4p•She3p] heterotrimers and thereby generate a transport-competent motor complex either for separate or co-transport of these two cargos.

P2049

Board Number: B568

Proinflammatory cytokine-induced Tight Junction remodeling through dynamic self-assembly of claudins.

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Tight Junctions (TJs) are dynamic, multiprotein intercellular adhesive contacts that provide a vital barrier function in epithelial tissues. TJs are remodeled during physiologic development and pathologic mucosal inflammation, and differential expression of the claudin family of TJ proteins determines epithelial barrier properties. However, the molecular mechanisms involved in TJ remodeling are incompletely understood. Using acGFP-claudin 4 as a bio-sensor of TJ remodeling, we observed increased Claudin 4 Fluorescence Recovery after Photobleaching (FRAP) dynamics in response to inflammatory cytokines.

Interferon gamma (IFN-gamma) and Tumor Necrosis Factor alpha (TNF-alpha) increased the proportion of mobile claudin 4 in the TJ. The claudin 4 PDZ (PSD95/Dlg/ZO-1) motif was required for cytokine-induced changes in dynamics. Upregulation of claudin 4 protein rescued these mobility defects and cytokine-induced barrier compromise. Furthermore, claudins 2 and 4 have reciprocal effects on epithelial barrier function, exhibit differential FRAP dynamics, and compete for residency within the TJ. These findings establish a model of TJs as self-assembling systems that undergo remodeling in response to proinflammatory cytokines through a mechanism of heterotypic claudin binding incompatibility.

P2050

Board Number: B569

From Microdishes to microniches. 3D microenvironmental control around single cells. Application to single cell apico basal polarization and lumenogenesis control.

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The influence of the microenvironment on cell behavior and fate is increasingly recognized as of key importance. It follows that new techniques to control the 3D environment around cells are key to understand the processes by which cells probe and respond to their microniches. We present here an approach that allow to transform microwells into artificial microniches where the chemical coating, rheological properties and topographical properties can be differentially controlled on the top, sides and bottom of the wells and assembled in a combinatorial way. This technique is also compatible with high and super resolution imaging that allows to probe the dynamics of cell cytoskeleton and regulatory proteins with unprecedented resolution down to the single molecule level in 3D. We exemplify how these bone fide artificial microniches can be used to induce full apico-basal polarization of single epithelial cells and as well as to control intercellular stresses driving the anisotropic growth of intercellular lumens. Other on going applications will also be briefly discussed.

Ref: Li *et al*, Nature Cell Biology, in revision

P2051

Board Number: B570

Apical lumen formation in single pluripotent human and mouse embryonic stem cells initiates via establishment of Rho-GTPase dependent intracellular apical membrane initiation site.

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Establishment of an apical surface and formation of a lumen is an essential process employed during morphogenesis of epithelial tissues and organs. We have found that single dispersed human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) organize apical and baso-lateral proteins at a peri-nuclear site prior to their first cell division. As the cell divides, this collection of proteins becomes the lumen between the two daughter cells; thus, it functions as an intracellular apical membrane initiation site (intra-AMIS). With further growth, these hESCs and hiPSCs form hollow cysts while maintaining the expression of NANOG and POU5F1. At the one-cell stage, the intra-AMIS structure contains highly distinct apical and baso-lateral compartments. A central apical domain is enriched for EZRIN and aPKC proteins (apical markers), surrounded by ring-shaped ZO1 protein (tight junction), a zone of RAB11 (early endosome) and GM130 (Golgi apparatus), as well as by junctional markers such as E-CADHERIN, β -CATENIN and β 1-INTEGRIN. This clearly shows the presence of intracellular baso-lateral domain in addition to apical. We show that the activation of Rho-mDia and Rac1-Arp2/3 signaling is essential for intra-AMIS formation and consequent patterning of the apical lumen, whereas inhibition of Rho-ROCK-myosin-II signaling promotes lumen formation. Other cell types, such as cultured Caco-2 (human colonic adenocarcinoma) and MDCK.2 (adult canine kidney) cells form intra-AMIS structures that evolve to lumens as well, suggesting that intra-AMIS structures are an important aspect of lumen formation in multiple settings and in multiple species. Like hESCs and hiPSCs, mouse embryonic stem cells (mESCs) also form intra-AMIS structures. Seeking an *in vivo* correlate to this phenomenon, we show that 4-day-old mouse blastocysts display intra-AMIS structures in primitive endoderm, trophoblast and epiblast precursor cells. Together, these data reveal that multiple cell types, including pluripotent human and mouse cells, have propensity to form lumina, and that the organization of the lumen-forming machinery begins with the formation of an intra-AMIS at the one-cell stage.

P2052

Board Number: B571

KLF4 controls epithelial stratification via non-canonical WNT5A signaling.

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Background: *Krüppel-like factor 4* (Klf4) is a DNA-binding transcriptional regulator highly expressed in gastrointestinal epithelia, specifically in regions of cellular differentiation. In the esophagus, Klf4 is expressed in differentiating cells of the suprabasal and superficial layers. Loss of *Klf4* perturbs normal squamous differentiation pathways and alters squamous epithelial stratification. Wnt5a is a non-canonical Wnt ligand down-regulated by gene expression analyses in *Klf4* conditional knockout mice. Wnt5a is important for the regulation of a variety of cellular functions, such as proliferation,

differentiation, migration, and polarity. We hypothesized that KLF4 controls esophageal epithelial stratification by direct transcriptional regulation of *WNT5A*. **Methods:** We used tissue specific gene ablation to delete *Klf4* in esophagus and sacrificed mice at 3 months of age for analyses. Primary esophageal keratinocytes were isolated for *in vitro* studies of gene regulation, cell migration, and stratification. **Results:** Tissue-specific *Klf4* knockout mice (*ED-L2/Cre;Klf4loxp/loxp*) had altered morphology of cells in the suprabasal and superficial layers. *Wnt5a*, a non-canonical Wnt ligand, was decreased by 3.0-fold on microarray, and we confirmed a 2.9-fold decrease by quantitative real-time PCR. These decreases in *WNT5A* were seen predominantly in the suprabasal layers of *ED-L2/Cre;Klf4loxp/loxp* mice. *Klf4* inducible knockdown with shRNAs in primary esophageal keratinocytes also produced a 30% decrease in *Wnt5a* expression levels. We found marked induction of *Wnt5a* promoter activity by KLF4 in primary esophageal keratinocytes using the luciferase reporter pGL4-*Wnt5a*. Moreover, we also demonstrated KLF4 binding to *WNT5A* by ChIP in human primary esophageal keratinocytes. Primary esophageal keratinocytes with *Klf4* knockdown had decreased polarity during cell migration, which was restored by recombinant *WNT5A*. Furthermore, we demonstrated that human primary esophageal keratinocytes with KLF4 knockdown had evidence of delayed cellular maturation, and defects in stratification in an organotypic 3D cell culture system. Treatment with recombinant *WNT5A* restored proper stratification in 3D cultures with decreased KLF4 expression. Conclusion: KLF4 is a key regulator of esophageal epithelial cell migration and stratification via *WNT5A*.

P2053

Board Number: B572

CRB3 restores epithelial cell shape and actin organization in cancer cells.

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BACKGROUND AND OBJECTIVES: Apico-basal polarity is a common feature of epithelial cells. Loss of the polarized architecture of epithelial cells is associated with many pathologies, including cancer. The transmembrane protein CRB3 is an important determinant of the apical domain in epithelial cells. Recently, CRB3 has emerged as a potential tumor suppressor, as it represses tumor growth and metastasis in animal models. However, the molecular mechanisms used by CRB3 to prevent tumor development and progression are unknown. Our goal is to decipher these mechanisms. **METHOD:** HeLa cells, which are devoid of endogenous CRB3, were transfected with a tagged form of CRB3, or truncations of CRB3. The phenotype associated with expression of these proteins was analyzed by biochemistry and cell biology techniques. **RESULTS:** Expression of CRB3 in HeLa cells results in a drastic change in cell morphology and a strong reorganization of the actin cytoskeleton. Cells also show tighter contacts with each other, suggesting an improvement of cell-cell junctions. These changes depend on CRB3 cytoplasmic tail, but do not require the extracellular domain. This indicates that CRB3 probably controls intracellular signaling pathways to trigger actin rearrangements. **CONCLUSION:** Expression of CRB3 in HeLa cells drives important structural changes similar to a mesenchyme-to-epithelial transition

in cancer cells. Expression of CRB3 in aggressive carcinomas could then prevent cancer progression and metastasis. Identification of the mechanisms controlled by CRB3 to limit tumor progression will likely highlight new targets for cancer treatment.

P2054

Board Number: B573

Alternative transcription of Amot80 and Amot130 in response to inflammation in breast cancer.

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The HIPPO signaling pathway is of central importance in controlling the growth of cells in tissues. Conversely, inactivation of HIPPO signaling underlies the progression and promotion of a multitude of cancer types, including breast cancers. HIPPO signaling inhibits the transcriptional co-activator YAP in response to cell-cell contacts and cell polarity. Reduced HIPPO signaling prompts the accumulation of YAP in the nucleus where transcription factors such as TEADs switch on pro-growth, -survival and -invasion programs. YAP is known to be inhibited by members of the Amot family of adaptor proteins. Because Amot proteins also bind the key polarity proteins that drive a differentiated morphology, this directly links YAP inhibition to formation of apical-basal polarity in epithelial cells. Because the Amot gene produces a 130 kDa form (Amot 130) that inhibits YAP and promotes cell polarity and an 80 kDa form (Amot 80), which acts as a dominant negative to promote YAP activation and cell growth, it is important to understand their differential regulation. The balance between expression of Amot80 versus Amot130 is shown to dictate whether breast cells will remain differentiated or undergo dysregulated growth. My analysis of breast tissues finds that total Amot is expressed weakly and localizes to the apical membrane in normal mammary cells. Conversely, Amot expression is significantly higher and localized to intracellular puncta (a hallmark of high Amot80 expression) in human breast cancers, especially in triple-negative forms of this disease. In hormone negative DCIS, Amot expression is high and peri-nuclear, specifically in the more disorganized regions adjacent to stromal areas flooded with inflammatory cells. This led us to examine how Amot transcription is modulated by inflammatory cytokines such as interleukin-6 (IL-6), a canonical activator of STAT3 that is commonly secreted in aggressive breast cancers. Phosphorylation of STAT3 (P-STAT3) by IL-6 activated Janus Kinase (JAK) receptors drives its nuclear accumulation and pro-growth transcriptional effects. Consistently I have found that IL-6 treatment of breast cancer cells increases Amot mRNA levels and immunoblot analysis indicates the differential induction of the Amot isoforms. The overall impact of these studies will be both the enhancement of our understanding of inflammation and growth control in breast cancer as well as a bioassay to measure Amot80 and Amot130 mRNA levels in human tumors. The lack of knowledge regarding the cross regulation between inflammatory signaling, HIPPO signaling, and cell polarity, allows this study to drive novel insights to the undisputed pro-tumorigenic impact of inflammation.

P2055**Board Number: B574****Farnesylated LKB1 regulates cell polarization and invasion through a 3D collagen microenvironment in a kinase-independent manner.**S. Wilkinson¹, A. Marcus²;¹Graduate Program in Cancer Biology, Emory University, Atlanta, GA, ²Hematology and Medical Oncology, Emory University, Atlanta, GA

The tumor suppressor LKB1 is a serine/threonine kinase that serves as a master regulator of cell polarity in epithelial tissue, and is mutated in 30% of non-small cell lung cancer tumors. LKB1 contains a C-terminal farnesylation motif, which allows for post-translational addition of a hydrophobic farnesyl group and insertion into the plasma membrane. Although LKB1 contains a farnesylation motif, the functional significance of this motif is largely unknown. We generated lung cancer cell lines stably expressing GFP-tagged: wildtype LKB1, a farnesylation motif mutant LKB1, and the various domains of LKB1, including the C-terminal domain (CTD, lacking kinase activity) and the CTD containing the farnesylation motif mutant, and then embedded spheroids of these cells in a 3D collagen matrix. Using confocal microscopy and live-cell imaging, we show that lung cancer cells re-expressing wildtype LKB1 acquire a unidirectional polarity, while cells expressing the farnesylation-mutant LKB1 are unable to polarize. Importantly, we show that 3D polarization is regulated through the LKB1 CTD alone, also in a farnesylation-dependent manner. These data indicate that farnesylated LKB1 promotes polarization during 3D motility through its C-terminal domain in a kinase-independent manner. We next sought to identify the role of LKB1 farnesylation in regulating invasion through the 3D collagen microenvironment. After acquiring a unidirectional polarity, cells re-expressing either wildtype LKB1 or the LKB1 CTD invade into the collagen microenvironment. Strikingly, we show that cells re-expressing LKB1 with the farnesylation motif mutation exhibit significantly reduced invasion into the collagen microenvironment, with the few cells invading exhibiting no polarization. Taken together, these data implicate LKB1 farnesylation as critical for promoting cell polarization and potentially providing a mechanism of cell invasion into the collagen microenvironment. This information has the potential to lead to novel information regarding the mechanisms of LKB1 farnesylation in regulating polarization and invasion, ultimately providing new insight into the tumor suppressive function of LKB1.

P2056**Board Number: B575****Adenomatous Polyposis Coli Regulates Epithelial Polarity and 3D Morphogenesis by Mediating FAK/Src Signaling.**A. Lesko¹, J. Prosperi^{1,2};¹Biological Sciences, Harper Cancer Research Institute, University of Notre Dame, South Bend, IN,²Indiana University School of Medicine, South Bend, IN

Adenomatous Polyposis Coli (APC) is a multi-functional protein that is lost or mutated in many epithelial cancers including breast, colorectal, and pancreatic cancer. Although APC is well known as a negative regulator of the Wnt/ β -catenin signaling pathway, it also binds to microtubules and polarity proteins, such as Dlg and Scribble, suggesting functions in regulation of epithelial polarity and cell migration. Our lab has previously determined that the mammary glands of *Apc*^{Min/+} mice demonstrate mis-regulation of epithelial polarity, exhibit early neoplastic changes, and develop more aggressive mammary tumors when crossed to the MMTV-PyMT model of breast cancer. Cells isolated from these tumors activated FAK/Src signaling. Our lab has also shown that APC knockdown in the Madin-Darby Canine Kidney (MDCK) model altered epithelial morphogenesis, resulted in inverted polarity in 3D culture, and up-regulated gene expression of epithelial membrane protein 2 (EMP2). While restoration of the b-catenin binding domain was unable to rescue the phenotype, introduction of either full-length or a c-terminal fragment of APC partially restored these phenotypes. The current studies investigate the Wnt-independent mechanisms by which APC regulates these processes using the MDCK model and primary mammary epithelial cells (MECs) isolated from *Apc* mutant mice. We hypothesize that the c-terminal fragment mediates FAK/Src signaling to regulate 3D morphogenesis and polarity. Treatment of APC knockdown MDCK cells with PP2, a Src kinase inhibitor, or AIIB2, an integrin inhibitor, eliminated the drastic cyst size changes produced by APC knockdown. Furthermore, inhibition of Src partially restored the polarity phenotype in cysts with APC loss. In addition, shAPC-MDCK cells exhibited increased cell migration indicating a role for APC in cell motility. Preliminary data demonstrates that treatment of shAPC-MDCK cells with PP2 and AIIB2 inhibits migration suggesting FAK/Src signaling as a possible mechanism by which APC mediates cell migration. Interestingly, EMP2 has been shown to bind integrin to activate FAK signaling suggesting an interaction in these signaling pathways. Future studies will aim to dissect the role of the c-terminal fragment and further devise the mechanism by which FAK/Src signaling and EMP2 play a role in APC regulating gene expression, cell migration, and polarity and 3D morphogenesis in MDCK cells. Investigating the interactions of APC with several targets such as those in the FAK/Src signaling pathway will help identify key players in the role of APC in Wnt-independent tumor development.

P2057**Board Number: B576****A cytoskeleton-based memory in chemotactic leukocytes.**H. Prentice-Mott¹, Y. Meroz², A. Carlson², M. Levine³, G. Charras⁴, L. Mahadevan², J. Shah¹;¹Harvard Medical School, Boston, MA, ²Harvard School of Engineering and Applied Sciences, Cambridge, MA, ³Biophysics, Harvard University, Cambridge, United States, ⁴London Centre for Nanotechnology, University College London, London, United Kingdom

The directional response of cells to asymmetric chemical environments involves a complex sequence of steps including: gradient sensing, internal polarization and migration. To understand how these modules act to direct cell motion requires quantitative measurements of the cellular response and quantitative control of the external chemical environment. We used microfluidics to generate confining microchannels in which the front and back of the cell can be independently exposed to separate chemoattractant concentrations, which are stable over time. Cells exposed to high, static differences with low background ($C_0=0$ nM, $\Delta C=100$ nM) exhibited persistent polarization, as assessed by PH-Akt asymmetry. Smaller differences ($C_0=0$ nM, $\Delta C=3$ nM) or increased background ($C_0=50$ nM, $\Delta C=50$ nM) resulted in fluctuations between polarized and unpolarized states. The direction of polarization was biased toward the higher concentration. When exposed to uniform concentrations of chemokine ($C_0=0, 3, 10, 100$ nM, $\Delta C=0$ nM), cells exhibited similar polarization fluctuations. Unexpectedly, the direction of polarization for all concentrations except $C_0=0$ was biased towards the initial direction of polarization, indicating a directional “memory”. To further test this, cells were exposed to a controlled dynamic environment, in which a uniform concentration ($C_0=10$ nM, $\Delta C=0$ nM) was first introduced, then changed to no chemoattractant ($C_0=0$ nM, $\Delta C=0$ nM) which caused most cells to depolarize. Subsequent reintroduction of the chemoattractant ($C_0=10$ nM, $\Delta C=0$ nM) after 2 minutes of no chemoattractant caused ~90% of the cells to re-polarize in the same direction as initially polarized. If the chemokine was removed for 10 minutes, this number decreased to ~70%. We measured the decay of internal cellular asymmetries during the no chemoattractant phase and observed dramatic differences in timescales for molecules such as PIP3 and Myosin light chain which were fast (10 seconds), versus the ERM protein moesin (5 minutes). Chemical disruption of the long-lived moesin (NSC668394) or microtubule (colcemid) structures during the no chemoattractant phase, resulted in decreased memory of polarization after reintroduction of the chemoattractant and washout of the drug (60% or 55%, respectively). We developed a phenomenological model incorporating two layers of polarization, a short-lived membrane layer (pm) and a long-lived cytoskeletal layer (pc). The cytoskeletal polarization is driven by the time-integrated pm , while the direction of the membrane polarization is driven by both pc and external concentration differences. This simple model reproduced the observed directional memory. Cells may use this directional memory to navigate the complex chemical environment found in tissues.

P2058**Board Number: B577****TorsinA operates as a membrane-regulated ring-shaped hexameric molecular chaperone within the nuclear envelope during centrosome orientation in migrating fibroblasts.**C.A. Saunders¹, E.M. Smith², Y. Chen², J. Mueller², G.G. Luxton¹;¹Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN, ²Department of Physics and Astronomy, University of Minnesota, Minneapolis, MN

The centrosome is oriented between the nucleus and the leading edge in many migrating cells and contributes to directional cell migration. Recent research shows that torsinA, a AAA+ ATPase found within the Nuclear Envelope (NE), is required for centrosome orientation during directional cell migration. AAA+ ATPases typically function as hexameric ring-shaped molecular chaperones to structurally remodel protein complexes by mechanically unfolding and translocating target proteins through a central pore. TorsinA is an unusual AAA+ ATPase in four ways: 1) it is found within the NE; 2) it is a monotopic membrane-associated protein; 3) it does not possess a recognizable target protein interaction domain; and 4) it lacks arginine fingers that are known to be important for ATP-hydrolysis and oligomerization. Therefore, the molecular mechanism of torsinA-mediated centrosome orientation is currently unknown. To begin to identify this mechanism, we tested the ability of different GFP-tagged torsinA constructs to rescue centrosome orientation in torsinA-null fibroblasts. We find that torsinA-mediated centrosome orientation requires ATPase activity and the ability of torsinA to form a hexameric oligomer with at least three active subunits. We identify two conserved hydrophobic residues (Y128 and Y147) in torsinA that are required for centrosome orientation as potential pore loops, which interact with and translocate target proteins in the central pore of AAA+ ATPases. To determine if torsinA can form hexamers, we developed z-scan Fluorescence Fluctuation Spectroscopy (FFS) in order to measure the oligomeric state of GFP-tagged proteins within the NE of living cells. Using this technique, we show that torsinA is on average a dimer within the NE while the deletion of the N-terminal membrane-association domain results in higher-order oligomeric states consistent with hexamerization. Finally, we show that torsinA lacking the membrane-association domain can still rescue centrosome orientation in torsinA-null fibroblasts. Taken together, our results suggest that torsinA operates as a hexameric ring-shaped molecular chaperone during centrosome orientation and that membrane-association negatively regulates torsinA oligomerization.

P2059**Board Number: B578****TorsinA is required for retrograde actin flow and TAN line assembly during rearward nuclear movement in migrating fibroblasts.**N.J. Harris¹, P. Willey¹, C.A. Saunders¹, G.G. Gundersen², G.G. Luxton¹;¹Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN, ²Department of Pathology and Cell Biology, Columbia University, New York, NY

Actin-dependent positioning of the nucleus at the cell rear is critical for the directional migration of several cell types. We have demonstrated that rearward nuclear positioning is mediated by linear arrays of nuclear envelope membrane proteins composed of nesprin-2G and SUN2, called Transmembrane Actin-associated Nuclear (TAN) lines, which couple the nucleus to actin cables undergoing retrograde flow. Currently, the molecular mechanism of TAN line assembly is unknown. Potential regulators of TAN line assembly are the torsin AAA+ ATPases that reside within the nuclear envelope. Recently, it was shown that torsinA interacts with the KASH domain of nesprin proteins within the nuclear envelope and is important for the localization of nesprins to the nuclear envelope. Since AAA+ ATPases typically function as molecular chaperones that structurally remodel protein complexes, we hypothesized that torsins may regulate the interaction of nesprin-2G and SUN2 during TAN line assembly. Here, we report that of the four mammalian torsins (torsinA, torsinB, tor2A, and tor3A) only torsinA is required for rearward nuclear positioning in migrating fibroblasts. Live imaging of fibroblasts where torsinA function has been inhibited reveals that the nucleus does not move directionally towards the cell rear. We find that torsinA localizes to TAN lines and is required for their assembly, supporting a role for torsinA in the regulation of nesprin-2G-SUN2 interactions. While torsinA inhibition does not negatively affect formation or organization of perinuclear actin cables, it does block retrograde actin flow. Taken together, our results suggest that torsinA controls both TAN line assembly and retrograde actin flow in migrating fibroblasts. Therefore, torsinA is a key regulator of cell polarity in during directional cell migration.

P2060**Board Number: B579****Armcx1, a potential gene of planar cell polarity regulation.**L. Knapp¹, P. Chen¹;¹Department of Cell Biology, Emory University, Atlanta, GA

Planar cell polarity (PCP) is essential in development, tissue morphogenesis, and cell migration. PCP refers to the coordination of cell polarity across a sheet of cells or tissues that utilizes communication between cells leading to a cytoskeletal response. The inner ear, specifically the cochlea's organ of Corti, is a model of PCP. Core PCP proteins have been implicated in coordinating sensory hair cell polarization, leading to uniform orientation of microvilli-derived stereocilia bundles in the organ of Corti. Specifically, membrane proteins Vangl2 along with Frizzled3 and Frizzled6 have been identified as core PCP proteins

required for PCP in the cochlea. How these core PCP proteins regulate morphological polarization is currently unknown. To explore and identify novel components of PCP that may bridge the core PCP proteins and cytoskeletal polarization, we performed a yeast two-hybrid screen to find proteins interacting with the cytoplasmic tail of Vangl2. Armadillo repeat-containing X-linked protein 1 (Armcx1) was identified as a Vangl2-interacting protein. Armcx1 contains armadillo repeats known to mediate protein-protein interactions in cell signaling and cytoskeletal morphogenesis. Down-regulation of Armcx1 mRNA has been linked to carcinomas in which PCP is perturbed. These findings support the hypothesis that Armcx1 may link Vangl2 to cytoskeletal polarization during PCP processes. To determine a possible role for Armcx1 in the PCP of the cochlea, we used in situ hybridization to determine whether its temporal and spatial expression pattern is consistent with a role in establishing PCP in the cochlea. We found that Armcx1 was expressed in the cochlear sensory epithelium, the organ of Corti, during establishment of PCP from embryonic (E) 14.5 to E 18.5 in mice. In addition, Armcx1 is also expressed in the vestibular sensory epithelia during PCP establishment. Together, its expression pattern, its interaction with known PCP proteins, and its associated cellular roles implicate Armcx1 as a potential regulator of PCP in vertebrates. We will further test whether Armcx1 interacts with Vangl2 to regulate cytoskeletal change and test its potential roles in PCP regulation.

P2061

Board Number: B580

Mitotic control of planar cell polarity by polo-like kinase 1.

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During cell division, polarized epithelial cells require mechanisms to ensure polarity and tissue integrity are preserved as they undergo dramatic structural rearrangements. Planar cell polarity (PCP), which relies on cell communication to align cell polarity across epithelial sheets, is particularly vulnerable during mitosis, as polarity defects can be propagated to neighboring cells. We previously discovered a novel mechanism in the skin epithelium that preserves PCP during mitosis whereby asymmetrically localized, cortical PCP proteins are internalized into endosomes, distributed equally to daughter cells, and recycled to the membrane where polarity is regained. The disassembly and restoration of PCP asymmetry must coincide precisely with cell cycle progression, but the mechanisms controlling PCP dynamics during mitosis are not known. Here we identify Plk1 as a master regulator of PCP dynamics during mitosis. Plk1 interacts with the core PCP component, Celsr1, via a conserved polo-box domain binding motif, localizes to mitotic endosomes and directly phosphorylates Celsr1's endocytic sorting signal. Phosphorylation activates the endocytic motif, allowing AP2 recognition and bulk recruitment of Celsr1 into clathrin-coated endosomes. Inhibition of Plk1 activity blocks PCP internalization, leading to perturbations in PCP asymmetry. Thus, Plk1-mediated phosphorylation of Celsr1 ensures PCP redistribution is precisely coordinated with mitotic entry.

P2062**Board Number: B581****Mitotic membrane turnover coordinates differential induction of the heart progenitor lineage.**C.D. Cota¹, B. Davidson¹;¹Biology, Swarthmore College, Swarthmore, PA

Matrix adhesion can polarize cells by stabilizing signaling enriched membrane domains. While it is known that dividing cells detach from the surrounding matrix and initiate extensive membrane remodeling, the in vivo impact of mitosis on adhesion-dependent signal polarization remains poorly characterized. Here we show that mitotic membrane turnover orchestrates adhesive polarization of heart progenitor induction. We investigate in vivo signaling dynamics in the invertebrate chordate, *Ciona intestinalis*. In *Ciona*, adhesion polarizes Fibroblast Growth Factor (FGF)-dependent induction of the heart progenitor lineage. Through targeted disruption and selective rescue of integrin based matrix adhesion in pre-cardiac founder cells, we show that adhesion promotes localized enrichment of FGF receptors by inhibiting mitotic internalization and degradation. Through mutational analysis of β -integrin subunits, we have experimentally defined the motif required for membrane stabilization and mitotic FGFR retention. Furthermore, targeted manipulations of Caveolin in the heart progenitor lineage indicate that Caveolin-rich membrane domains function downstream of polarized cell-matrix adhesion to retain FGFRs during mitotic rounding. These results support a revised model of adhesion-dependent asymmetric fate induction in which mitotic membrane turnover resets historic, pre-mitotic receptor distribution according to contemporaneous adhesive cues.

P2063**Board Number: B582****SONIC HEDGEHOG SIGNALING CONTROLS GRANULE NEURON MIGRATION IN A RAS-RAF DEPENDENT PATHWAY.**T. Ong¹, D. Solecki²;¹Developmental Neurobiology, St. Jude Children's Research Hospital, Memphis, TN, ²developmental neurobiology, St. Jude Children's Res Hosp, Memphis, TN

Background: Neuronal progenitors in the brain are born in sites known as germinal zones (GZ) where they undergo proliferation and differentiation before migrating long distances to home to their final positions. Developmental cues that control this transition are tightly regulated to ensure proper brain morphogenesis as dysregulation leads to brain malformations, mental retardation and cancer. A major challenge in the field of developmental neurobiology is to delineate the molecular underpinnings that regulate this developmental program. In the cerebellum, cerebella granule neurons (CGN) that fail to exit the external germinal layer (EGL) due to aberrant sonic hedgehog (Shh) signaling continue to proliferate and give rise to Shh driven medulloblastoma (MB). **Results:** Our laboratory have previously

reported that the E3 ubiquitin ligase, Siah2, blocks CGN migration by negatively regulating Par3, a component of the Par complex that is required to recruit the junctional adhesion molecule, JamC to the membrane to initiate cell-cell contacts for GZ exit. Preliminary data show that Shh signaling maintains Siah2 protein expression in a Ras-Raf dependent pathway. We also show a direct correlation between protein levels of active Ras (H and N isoforms) and Siah2, where their levels decrease from post-natal days (P), P4 to P15 respectively. Interestingly, these protein levels remain high in Ptc+/-P18-/- MB cells. In line with these findings are functional studies that show that activation of Shh signaling or gain of function of the Ras-Raf pathway in granule neuron progenitors (GNP) impairs their GZ exit which can be rescued by exogenous expression of Par3 or inhibition of Siah2 function, respectively. **Conclusion:** We propose that Shh signaling negatively regulates Par3 in a Ras-Raf-Siah2 dependent manner, hence blocking CGN GZ exit.

P2064

Board Number: B583

Rab35-dependent axon elongation is negatively regulated by p53-related protein kinase (PRPK).

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The appropriate function of the nervous system relies on the neuronal polarized morphology. Axon elongation is a crucial process through neuronal final shape is achieved, and a mechanism sustained mainly by cytoskeletal dynamics and intracellular trafficking. We previously described that microtubule-associated protein 1B (MAP1B) is required for proper axon outgrowth. The aim of this work is to describe novel MAP1B-interacting proteins which could be involved in axon elongation. We carried out a yeast two-hybrid experiment using MAP1B light chain (LC1) as bait, and detected PRPK as one of the LC1-interacting partners. PRPK overexpression in hippocampal embryonic neurons in culture significantly reduces their axonal length. PRPK-induced phenotype can be rescued by Rab35 overexpression, suggesting that both proteins are functionally related. Noteworthy, axonal defects found in MAP1B knock-out neurons can be recovered by overexpression of either PRPK kinase dead mutant or Rab35 constitutively active. These evidences show that the MAP1B-interacting partner PRPK negatively regulates axon elongation in vitro, in a Rab35-dependent manner.

This work was supported by Fondecyt-1140325 (to CG-B)

P2065**Board Number: B584****Oligodendrocyte-innate programs dictate cell shape changes required to form myelin sheaths and account for variation in lengths of CNS myelin sheaths.**M.E. Bechler¹, L. Byrne¹, C. French-Constant¹;¹MRC Centre for Regenerative Medicine, The University of Edinburgh, Edinburgh, Scotland

Myelin internodes are specialized extensions of glial cell plasma membrane spirally wrapped around axons to create a multilamellar sheath. The length of these internodes regulates the speed of axonal signal propagation. This length usually correlates with underlying axon diameters, suggesting the hypothesis that axonal cues regulate the internode properties and instruct the remarkable changes in cell shape required to form a sheath. However, despite decades of work to identify instructive cues, no axonal molecule has been shown to be required for central nervous system (CNS) myelination. Using primary oligodendrocytes cultured with inert microfibers, we tested the alternative hypothesis that myelin sheath formation is an innate property of the oligodendrocyte refined by, but not requiring, axonal signals. Supporting this hypothesis, oligodendrocytes produce internodes of physiological lengths on these microfibers, with compact multi-layered myelin membranes. This oligodendrocyte-innate program can be adapted by specific axonal molecules, with independent regulation of the number and length of internodes. This is distinct from Schwann cells that require axonal cues to differentiate and form compact myelin sheaths. Oligodendrocytes also inherently respond to the physical cue of microfiber diameter, increasing internode length with increased diameters. Surprisingly, we find that oligodendrocytes isolated from distinct CNS regions have differing innate internode lengths. This mirrors the variation in internode lengths found in vivo: spinal cord oligodendrocytes produce longer sheaths than those from cortex. These results indicate the cell shape changes during myelin internode formation are inherently controlled by oligodendrocytes and reveal previously unsuspected differences between oligodendrocytes from different regions of the CNS.

P2066**Board Number: B585****ROCK1 and 2 differentially regulate actomyosin organization to determine cell polarity in fibroblasts and neurons.**K.A. Newell-Litwa¹, M. Badoual², H. Asmussen¹, H. Patel¹, L. Whitmore¹, A.R. Horwitz¹;¹Cell Biology, University of Virginia, Charlottesville, VA, ²Laboratoire IMNC, Univ. Paris-Sud, Orsay, PA

RhoGTPases give shape to the cell by organizing the underlying actin cytoskeleton. As such, they are implicated in polarity underlying normal and cancer cell migration, epithelial polarization, and even the subcellular organization of the polarized dendritic spine, which supports learning and memory. In particular, RhoA-mediated signaling activates non-muscle myosin IIB (MIIB) by di-phosphorylating its regulatory light chain to form actomyosin filament bundles that define the sides and rear of migrating

cells. RhoA also inactivates Rac-driven actin polymerization, via an unclear mechanism, thus segregating the initiation of actin polymerization to the front of the cell to create the leading edge. However, how RhoA coordinates these two processes to drive polarity is unknown. ROCK is the major RhoA effector kinase that activates MIIB to create the cell rear. Yet, it is unclear which ROCK isoform, ROCK1 or ROCK2, is responsible for MIIB activation and Rac inactivation. We examined whether ROCK1 and 2 similarly or separately regulate MIIB activation and Rac inactivation in both front-back migratory cell polarity and dendritic spine polarity. For both systems, we observed a specific role for ROCK1, but not ROCK2, in the di-phosphorylation of myosin regulatory light chain on Thr18 and Ser19. This ROCK1-driven MIIB activation was also necessary for proper microtubule organization. In contrast, ROCK2, but not ROCK1, specifically attenuated Rac activity underlying the formation of the leading edge of the migratory cell and the spine head of the synapse. We hypothesized that ROCK2 regulates Rac activity through a negative feedback mechanism with the actin remodeling protein, cofilin. While Rac activation normally inactivates cofilin by Ser3 phosphorylation; knockdown of ROCK2 decreased levels of Ser3-phosphorylated cofilin. The loss of phosphorylated cofilin resulted in smaller nascent adhesions, which signal through Rac to drive membrane protrusion. However, cofilin inactivation by expression of phosphomimetic cofilin S3D restored adhesion maturation and rescued the morphology of ROCK2-deficient cells. Similarly, cofilin inactivation regulated the size of post-synaptic densities within the spine head. Thus, ROCK1 and ROCK2 regulate distinct molecular pathways downstream of RhoA, and their coordinated activities drive polarity in both cell migration and synapse formation. These results also suggest that the ROCK inhibitor, Y-27632, which indiscriminately targets both isoforms, is inhibiting two different pathways.

P2067

Board Number: B586

Contribution of Reactive Oxygen Species to the Establishment of Hippocampal Neuronal Polarity.

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Basal production of Reactive Oxygen Species (ROS) by the NADPH (NOX) complex contribute to LTP, memory consolidation and neuronal actin cytoskeleton dynamics. The Rho GTPases Rac1 and Cdc-42 and the actin cytoskeleton are essential for the development of the axo-dendritic compartments. Acquisition of polarity is intimately related with neuronal functions. However, the contribution of ROS during neuronal polarity has not been explored. In this work, we propose that physiological ROS produced by NOX complex contributes to the acquisition of neuronal polarity and axonal growth.

Hippocampal neurons from rat brain embryos (E18.5) were cultured for different times to evaluate whether ROS are involved in the establishment of neuronal polarity. Epifluorescence microscopy was used to assess development of neuronal polarity and axonal length. ROS content and actin dynamics were visualized using the genetically encoded biosensors Hyper and Lifeact, respectively. In addition, FRET biosensors were used to measure local activity of Rac1 and Cdc-42 after NOX inhibition.

The NOX subunits gp91^{phox}, p22^{phox}, p47^{phox} and p67^{phox} were detected in stage 2 and stage 3 hippocampal neurons. NOX complex inhibition (apocynin, gp91ds-tat and transfection of p22^{phox} P156Q (DNp22^{phox})) delayed polarization of hippocampal neurons and decreased axonal length. In addition, DNp22^{phox}-transfected neurons showed a decrease in the number, length and life-time of axonal filopodia. Moreover, the activities of Rac1 and Cdc-42 were also decreased after DNp22^{phox} expression.

Our results suggest that ROS produced by NOX complex contributes to the acquisition of neuronal polarity by regulating actin cytoskeleton. We propose that physiological levels of ROS may be necessary for polarization and maturation of hippocampal neurons.

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P2068

Board Number: B587

In vitro interaction of drugs with canalicular lipid transporters.

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Background and aims: Canalicular bile formation is maintained by the simultaneous activities of three ABC-transporters: BSEP (Bile Salt Export Pump or ABCB11), ABCB4 (or MDR3) and ABCG5/G8. Dysfunctions in the biliary cholesterol–bile salt–phospholipid secretion (e.g. an increased ratio of cholesterol to bile salts or phospholipids) lead to cholestasis or result in crystallization of cholesterol followed by cholelithiasis. The aim of this project is the development of a cell-based and polarized model system to test the hypothesis that some drugs may induce cholestatic liver disease by specifically interfering with biliary lipid secretion and to identify such inhibitors/stimulators.

Methods: Stably transfected LLC-PK1 cell lines (overexpressing human NTCP, BSEP, ABCB4 and ABCG5/G8) were cultured in the Transwell® system. Detection and polar localisation of the transport proteins was accomplished by immunohistochemistry and Western blots. Functionality and direction of transport were verified using radioactively (e.g. 3H-taurocholate and 14C-cholesterol) or fluorescently (NBD-phosphatidylcholine) labelled substrates. The substrates were added to the basolateral or apical compartments and the amounts transported in the opposite compartment were measured. Albumin was used as an acceptor to stimulate efflux. Lipid secretion into the apical compartment was also chemically analysed by thin-layer-chromatography.

Results: Western blots and immunohistochemistry indicate that the transfected LLC-PK1 cells overexpress all three ABC transporters and that NTCP is overexpressed in the basolateral membrane while BSEP, ABCB4 and ABCG5/G8 are overexpressed in the apical membrane, as is the case in human hepatocytes. Functional transport assays demonstrate that the model cell line displays a vectorial transport of bile salts and phosphatidylcholine from the basolateral to the apical compartment. First experiments with the antimycotic agent itraconazole show slight changes in phosphatidylcholine secretion but a strong induction of ABCB4 expression.

Conclusions: The antimycotic agent itraconazole does not significantly change the amount of phosphatidylcholine secreted but strongly induces ABCB4 expression indicating that itraconazole indeed inhibits phosphatidylcholine secretion in our assay system if the protein amount is considered. This finding is in line with previously published data. Taking this result together with the vectorial transport bile salts and phosphatidylcholine, we have successfully established an in vitro model for canalicular bile salts and lipid secretion. Consequently, we plan to use this model to characterize the effect of additional drugs suspected to interfere with canalicular bile salts and lipid transporters.

P2069

Board Number: B588

The apical polarity of Na⁺,K⁺-ATPase in ARPE-19 cells is regulated by the expression of the β 2 subunit.

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The Na⁺, K⁺ ATPase or sodium pump plays a very important role in the maintenance of epithelial phenotype. This protein is always polarized in epithelia and is crucial for creating and maintaining the electrochemical gradient of Na⁺ and K⁺ as well as the cell volume and osmolarity. In most epithelia, the pump is located in the lateral domain. We demonstrated that the β 1-subunit of the Na⁺, K⁺ ATPase plays an important role in the polarization mechanism as it interacts directly, in the extracellular space with another β 1 subunit localized on a neighboring cell and thus stabilizes the pump in the lateral membrane. However, in the retinal pigment epithelium (RPE) the sodium pump is located at the apical domain. The mechanism of polarization in this epithelium is still unknown. Our working hypothesis is that the apical polarity of the pump in RPE cells is regulated by its β 2 subunit. We got evidences that relate the apical localization of the pump in RPE with the expression of the β 2 subunit, and we are looking for the mechanism by which β 2 subunit regulates the pump polarity. Results: ARPE-19 cells cultured for up to 6 weeks in the presence of ITS acquire the RPE phenotype and express the sodium pump in the apical domain (process termed re-morphogenesis). WB and IF analyses show that the β 2 isoform detected at the apical domain is co-expressed with the α 2 subunit. In order to find out if the β 2 expression is regulated on a transcriptional or translational level, we analyzed as function of time, the mRNAs level of the β subunit isoforms by RTqPCR and the protein expression level by WB. We observed that in the presence of ITS, β 2 mRNAs are increased during re-morphogenesis, as well as the protein level. Silencing the β 2 expression by siRNAs, reduce the apical expression of the pump as gauged by the apical localization of the α 2 subunit. Our results suggest that the apical localization of the pump in ARPE-19 cells depends on the expression of the β 2 isoform and that it is regulated on a transcriptional level. Accordingly, we suggest that the transcription factor Sp1 is involved as it is also expressed in ARPE-19 cells and its amount increases during re-morphogenesis. We therefore expect that silencing Sp1 by siRNA will inhibit the apical localization of the pump (α 2 β 2 complex).

P2070**Board Number: B589****A spatial switch in H,K-ATPase trafficking by *Helicobacter pylori* VacA-elicited phosphorylation of ACAP4.**X. Ding¹, X. Liu², W. Yao², L. Chen³, Y. Zhang¹, X. Yao⁴;¹Medicine, Beijing University of Chinese Medicine, Beijing, China, ²Physiology, Morehouse School of Medicine, Atlanta, GA, ³Beijing University of Chinese Medicine, Beijing, China, ⁴Cellular Dynamics, University of Science Technology of China, Hefei, China

Many cellular processes are orchestrated by dynamic changes in the plasma membrane to form membrane projections and endocytic vesicles. Small GTPase ARF6 is involved in coupling actin dynamics to trafficking of vesicular membranes. Our study demonstrated that ACAP4, ARF6 GTPase-activating protein, governs polarized H,K-ATPase trafficking during parietal cell secretion (J. Biol. Chem. 2010. 285, 18769-80). *Helicobacter pylori* persistently colonize the human stomach and have been linked to atrophic gastritis and gastric carcinoma. Our early study revealed that VacA disrupts apical membrane-cytoskeletal interactions and perturbs parietal cell secretion (J. Biol. Chem. 2008. 283, 26714-25). VacA binds to tyrosine phosphatase PTP- ζ which leads to gastric ulcer (Nature Genetics. 2003. 33, 375-381). However, it is unclear how PTP- ζ operates its downstream signaling events in parietal cells. Here we show that PTP- ζ interacts with ACAP4 and VacA treatment enhances ACAP4 phosphorylation at Tyr733 which rewires cellular signaling for aberrant proton secretion. Our biochemical characterization and cellular imaging analyses show that VacA-elicited phosphorylation of Tyr733 results in re-distribution of ACAP4 to the basolateral membranes. Surprisingly, persistent expression of phospho-mimicking ACAP4 results in recruitment of H,K-ATPase and v-SNAREs to the basolateral domains. The large basolateral expansion elicited by VacA is predicted to recruit membranes from sources not normally targeted to that surface. Significantly, the phosphorylation of Tyr733 was correlated to hypochlorhydria phenotype of clinical VacA-positive patients. In addition, expression of non-phosphorylatable Y733F mutant in ACAP4 knockout mouse parietal cells resumes apical secretion of proton, suggesting that VacA rewires ACAP4 signaling axis by repolarizing parietal cell secretion at basolateral membrane. The results present a previously undefined mechanism by which microbe-host interaction orchestrates a spatial switch of membrane trafficking in gastric parietal cell secretion.

P2071**Board Number: B590****Large, low-complexity MLT1 protein is associated with unique, eyespot-associated microtubules in the green alga *Chlamydomonas reinhardtii*.**T.M. Mittelmeier¹, M.D. Thompson¹, M. Lamb², C.L. Dieckmann¹;¹Molecular and Cellular Biology, University of Arizona, Tucson, AZ, ²Biology, University of Puget Sound, Tacoma, WA

The photosensory eyespot of the unicellular green alga *Chlamydomonas* is found at the equator of the cell in a unique position relative to the two anterior basal bodies and flagella. In response to light, eyespot-initiated signals lead to changes in flagellar stroke patterns and swimming direction, such that the incident light intensity is optimal for photosynthesis. The eyespot comprises components in the plasma membrane, cytoplasm, and chloroplast envelope membranes, and in carotenoid-rich lipid granules associated with thylakoid membranes. Following cell division, the eyespot is assembled *de novo* in each daughter cell adjacent to the D4 microtubule rootlet, one of four rootlets that extend from the basal bodies. During assembly, localization of one or more eyespot components is directed by the D4 microtubules. *mlt1* (multi-eyed) mutant cells contain additional eyespots that are mis-localized to more anterior positions, and localization of both major eyespot photoreceptor proteins is delayed. We used polyclonal antibodies to identify wild-type MLT1 on Western blots and in fixed cells by immunofluorescence. The >300 kDa protein was localized along the D4 rootlet, but not the other three rootlets. MLT1 is absent in *mlt1-1* cells, which have a nonsense mutation near the middle of the *mlt1* coding sequence. Similar to the *Arabidopsis* microtubule-binding protein BPP1 (basic proline-rich protein 1), MLT1 is of low complexity and proline-rich, and has three exclusively basic regions. We are testing the hypothesis that binding of MLT1 to rootlet microtubules via its basic region(s) stabilizes the microtubules and/or promotes microtubule-guided trafficking of eyespot components. MLT1 is also absent in *mlt2-1* mutant cells, which have a phenotype similar to but distinct from *mlt1* cells. Sequencing of the *mlt2-1* genome identified the *MLT2* gene, predicted to encode a large, low-complexity, leucine-rich protein. While eyespot localization is dependent on MLT1 and MLT2, the integrity of the ordered membrane layers of the assembled eyespot is dependent on the MIN1 protein which has an N-terminal C2 domain and a C-terminal LysM domain. Extracellular LysM domains are known to bind bacterial or fungal cell wall components, but the function(s) of intracellular LysM domains is unknown. We observe that cells with changes in highly conserved residues, modeled to be on the surface of the folded MIN1 LysM domain, have “fragmented” eyespots. The data are suggestive that the MIN1 LysM domain is involved in specific interactions that maintain the close apposition of eyespot membrane layers. Further analyses of eyespot proteins will aid our understanding of the functional breadth of a number of widely distributed protein motifs. Support: NSF MCB-1157795 to CD.

P2072**Board Number: B591****Congenital Tufting Enteropathy, a new model for studying intestinal villous morphogenesis.**

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Molecular mechanisms underlying the maintenance of the intestinal villous epithelial layer homeostasis are largely unknown. Congenital Tufting Enteropathy (CTE), (MIM #613217) a rare human cause of intestinal failure, constitutes an interesting working model. It is characterized by aberrant focal stacks of multiple layers of enterocytes, named « tufts » and is due to mutations in the EPCAM or SPINT2 genes. Our study aimed at understanding the cellular functions of EPCAM and SPINT2 in enterocytes and in villous morphogenesis. EPCAM and SPINT2 are located at the lateral membranes and the terminal web in human enterocytes. We analyzed either duodenal biopsies of CTE patients, or stable Caco2 clones depleted for EPCAM or SPINT2. Ultrastructural analyses were performed using transmission electron microscopy. Cell organization, cell differentiation and polarity markers were tested using immunohistology and confocal microscopy analyses. Our results clearly show specific defects in intestinal cell polarity and adhesion in the mutant enterocytes: microvillus atrophy, mislocalization of brush border components, and weakened cell-cell junctions. Although Caco2-depleted cells display a loss of apico-basal polarity, they still formed epithelial monolayers in classical 2D cultures. We thus generated 3D culture supports that recapitulate villus topology and constraints. Placing mutant human enterocytes on synthetic villus cultures revealed strong perturbations in the maintenance and dynamics of the epithelial monolayer, mimicking the tufts observed in CTE patient mucosa. We demonstrated that CTE disorder is characterized by the loss of enterocyte polarity and differentiation. As a consequence, correct cell dynamics along the villous architecture is impaired in mutant enterocytes.

P2073**Board Number: B592****Spontaneous Cdc42 polarization independent of GDI-mediated extraction and actin-based trafficking.**F.O. Bendezu¹, V. Vincenzetti¹, D. Vavylonis², R. Wyss³, H. Vogel³, S.G. Martin¹;¹Department of Fundamental Microbiology, University of Lausanne, Lausanne, Switzerland, ²Department of Physics, Lehigh University, Bethlehem, PA, ³Institut des Sciences et Ingénierie Chimiques (ISIC), Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

How a cell polarizes growth is a fundamental biological question. The conserved Rho-type GTPase Cdc42 is a central player in this process. Activated GTP-bound Cdc42 determines the zone of growth by interacting with effectors necessary for the targeting of secretory vesicles. It is not clear, however, how cells spontaneously polarize to establish a zone of active Cdc42. This symmetry-breaking event is thought to require dynamic Cdc42 recycling off and on the plasma membrane by GDI (Guanine nucleotide Dissociation Inhibitor)-mediated membrane extraction and actin-based vesicle trafficking. Here, we describe the construction of a functional, internally tagged Cdc42-mCherry sandwich (SW) fusion expressed from the endogenous *cdc42* locus in fission yeast. Using pharmacological inhibitors and mutant backgrounds, we find Cdc42 dynamics and spontaneous polarization is maintained largely independent of these proposed recycling pathways. Further strengthening these findings, an engineered Cdc42 allele targeted to the membrane independently of these recycling pathways by an amphipathic helix supports viability and polarizes spontaneously to multiple sites in both fission and budding yeasts. Using Fluorescence Recovery After Photobleaching (FRAP) and Fluorescence Correlation Spectroscopy (FCS), we find that Cdc42 is highly mobile at the plasma membrane. However, mobility is slower at sites of active Cdc42-GTP, which correspond with areas of total Cdc42 accumulation. By contrast, an engineered near-immobile transmembrane domain-containing Cdc42 allele supports viability and polarized activity, but does not accumulate at sites of activity. We propose that Cdc42 activation, enhanced by positive feedbacks, leads to its local accumulation by capture of fast-diffusing inactive molecules at the plasma membrane.

P2074**Board Number: B593****A minimal system to establish microtubule-based cell polarity in vitro.**N. Taberner¹, P. Recouvreux², S. Roth¹, M. Dogterom¹;¹BionanoScience, TU Delft, Delft, Netherlands, ²IBDM - Aix Marseille University - CNRS, Marseille, France

From yeast to fibroblasts, many cell types have a defined polarity that allows them to directionally move, grow or divide. This polarity is typically defined by a polarized distribution of proteins at the cell cortex. We are interested in the emergence of polarity in systems where microtubules are directly involved in its establishment and maintenance by delivering polarity markers to the plasma membrane. We developed two in vitro systems that allow for microtubule-based delivery of proteins to bio-mimetic

cortices. These systems, consisting of elongated micro-fabricated chambers or emulsion droplets, allow for dynamic microtubules to self-assemble and organize in response to interactions with the chamber or droplet boundaries.

In micro-chambers, single events of microtubule-based delivery to a wall are imaged and quantified. Our experiments show that clustering of proteins at microtubule tips (fission yeast's mal3, kinesin tea2, and tip1) enhances prolonged docking of proteins to the wall receptors as opposed to non-clustering proteins (EB analog protein mal3). Moreover, pre-docked clusters at the wall can capture growing microtubule tips enhancing repeated deliveries at the same spot. These observations are very similar to the observation of clustering of polarity markers in living fission yeast cells.

With elongated emulsion droplets the global emergence of polarity in a closed system can be assessed under conditions where proteins can additionally diffuse within the lipid boundaries. With this minimal system we aim to establish the minimal mechanism by which microtubules can establish and maintain cell polarity in living cells. In parallel, we performed experiments in living yeast cells, which suggest that a simple artificial protein that combines membrane affinity with microtubule tip affinity is in principle enough to establish (but not maintain) polarity.

P2075

Board Number: B594

Role of a clutch molecule shootin1 in laminin-induced axon formation.

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Neuron requires correctly oriented axon formation to transmit their signals to appropriate target cells. Axon is directed by asymmetric presentation of soluble and adhesion axon guidance molecules. For example, an extracellular matrix protein laminin contact directs neuronal polarization of retinal ganglion cells to basal lamina in vivo [Owen et al, *Neuron*, 2011]. The growth cone plays a key role in neurite extension to form axon. However, how does laminin affect growth cone and induce directional axon formation remains elusive. Shootin1 mediates a linkage between F-actin retrograde flow and cell adhesion molecule L1-CAM as a clutch molecule at the growth cone and induces traction force for axonal outgrowth [Shimada et al, *J. Cell Biol.*, 2008., Toriyama et al, *Curr. Biol.*, 2013]. As L1-CAM extracellularly interacts with laminin [Hall et al, *J. Neurochem.*, 1997], we hypothesized that axon is induced on laminin by shootin1-mediated linkage between F-actin and L1-CAM. To examine this possibility, we analyzed effect of shootin1 RNAi on directional axon formation of hippocampal neurons cultured on substrates patterned with stripes of laminin and poly-D-lysine. The rate of neurons that formed axon on laminin was significantly decreased by shootin1 RNAi as compared with control. Next, we measured retrograde flow rates of mRFP-actin and EGFP-shootin1 speckles. As L1-CAM binds with laminin, retrograde flow of F-actin and shootin1 would be negatively regulated by laminin on the extracellular substrate. Retrograde flow rates of mRFP-actin and EGFP-shootin1 speckles in the axonal

growth cone on laminin were markedly slower than those on PDL. Because retrograde flow rates of mRFP-actin and EGFP-shootin1 speckles were decreased in the axonal growth cone on laminin, traction force that the axonal growth cone generates would be positively regulated on laminin. To examine this possibility, we performed traction force microscopy. Average stress under the growth cone on laminin was larger than those on PDL. Together, our results suggest that the clutch system involving shootin1 and L1-CAM senses an adhesion cue from laminin and produces traction force for axon formation.

Neuronal Cytoskeleton 2

P2076

Board Number: B596

Somal translocation of Retinal Ganglion Cells: a new mode of microtubule involvement in neuronal migration.

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Neuronal migration is a key step during brain development and defective neuronal migration can lead to cognitive impairments. Importantly, neuronal migration modes differ depending on tissue context and migratory distances covered. Despite this broad variety however, most mechanistic studies have so far focused on radial glial-guided migration

To get insights into additional neuronal migration modes we investigate the kinetics and molecular mechanisms responsible for retinal ganglion cell (RGC) migration in zebrafish. RGCs are the earliest born neurons in the retina and migration from their apical birthplace to the most basal neuronal layer spans about 50µm. It was proposed that RGCs move via somal translocation but the exact molecular mechanisms of migration are still elusive.

We image migratory processes in the intact embryos using light sheet as well as confocal microscopy at high temporal-spatial resolution. This approach revealed that RGC migration occurs unidirectionally and migratory speeds reach 20 µm/h. To exclude the possibility that somal translocation is a passive process we inhibited nuclear movements of remaining progenitor nuclei. This treatment did not significantly impair RGC migration arguing that it is an active process depending on force generation within the cells. Interestingly, we observe that the centrosomes as well as the Golgi follow the migrating soma during RGC translocation instead of leading it as seen in glia-guided migration. Nevertheless, like in glial-guided migration, both, microtubules and dynein play a role in RGC displacement. Microtubules and dynein are apically enriched during migration, indicating that the force to move cells is generated BEHIND nucleus and soma. This finding revealed the possibility that microtubules push the nucleus, the bulkiest organelle of the cell, in the direction of basal migration and/or prevent re-displacements by acting as an apical barrier. The observation that basal process attachment is crucial for efficient migration underlines this exciting possibility. We currently test this hypothesis further using laser ablation approaches to

explore whether apical and basal processes feature different mechanical properties. We also test the possibility that dynein is important to give rigidity to apical microtubules and thereby stabilizes the apical process.

Overall our study aims to gain a basic understanding of the molecular mechanism involved in somal translocation that will most likely also apply in brain regions beyond the retina. It furthermore shows that neurons can achieve successful migration differently in different scenarios and underlines the fact that neuronal migration has many different flavours several of which still await further exploration.

P2077

Board Number: B597

Synaptotagmin-1's Promotion of the Formation of Axonal Filopodia in developing Neurons is associated with Calcium Flux.

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Synaptotagmin-1 (syt1), a Ca²⁺-binding protein that functions in regulation of vesicle exocytosis at the synapse, is expressed in many types of neurons well before synaptogenesis begins both in vivo and in vitro. We recently demonstrated (Greif et al, 2013) that syt1 plays a role in regulating axon branching and filopodial dynamics in developing embryonic chick forebrain neurons. Overexpression of syt1 increased the formation of axonal filopodia and branches. Conversely, knockdown of syt1 decreased the numbers of axonal filopodia and branches. The mechanism by which syt1 exerts its influence on axon development is unknown. In order to investigate whether syt1 accomplishes its effects on filopodia using a mechanism similar to its role at the synapse, we manipulated Ca²⁺ levels using calcium blockers and calcium ionophores. A 15-minute exposure to the calcium ionophore, A23187, significantly increased axonal filopodia; blockade of calcium channels using La³⁺ or Cd²⁺ decreased filopodia number. These findings are consistent with the well-defined role of Ca²⁺ in filopodial dynamics. Overexpression of syt1 using adenovirus-mediated transfection was coupled with La³⁺ treatment to examine whether blocking Ca²⁺ influx would reverse Syt1-mediated increases in axonal filopodia. Embryonic chick forebrain neurons were transfected with a syt1-YFP construct or GFP. After 48 hrs, cells were treated with La³⁺ or vehicle for 15 minutes. Treatment with La³⁺ reversed the increase in filopodia stimulated by syt1 overexpression. Taken together these data suggest that syt1 regulates the formation of axonal filopodia using its Ca²⁺-binding functions, prior to engaging in its conventional functions at the synapse. Future experiments involve site-directed mutagenesis of syt1's C2 domains, to directly study our hypothesis. This research was supported by grants from Bryn Mawr College.

Reference: Greif, K.F., N. Asabere, G. J. Lutz and G. Gallo. Synaptotagmin-1 promotes the formation of axonal filopodia and branches along the developing axons of forebrain neurons, Dev Neurobiol. 2013 Jan; 73(1):27-44.

P2078**Board Number: B600****Fidgetin is a novel microtubule-severing protein that controls neuronal morphology by balancing the stable and labile components of the axonal microtubule array.**L. Leo¹, A. Matamoros², W. Yu³, D. Marena⁴, D. Sharp⁵, P.W. Baas³;¹Neuroscience, Drexel University College of Medicine, Philadelphia, PA, ²Neuroscience/Molecular and Cell Biology and Genetics, Drexel University College of Medicine, Philadelphia, PA, ³Department of Neurobiology and Anatomy, Drexel University College of Medicine, Philadelphia, PA, ⁴Biology, Drexel University, Philadelphia, PA, ⁵Albert Einstein College of Medicine, Bronx, NY

The dynamicity of the axonal microtubule (MT) array is defined by stable and labile components. All of the MTs in the axon have a stable domain, and most have a labile domain. MT-severing proteins are important for regulating the axonal MT array by transforming one MT into two, for example to increase the number of MTs at sites of impending branch formation. In order to accomplish this, katanin and spastin (the best studied of the MT-severing proteins) preferentially break MTs in their stable domains, so that each of the two daughter MTs has its own stable domain from which a new labile domain can grow. Theoretically, a MT-severing protein that preferentially breaks MTs in their labile domains would have a very different effect. Specifically, such a severing protein would result in one MT with a stable domain and one without, the latter of which would rapidly and completely depolymerize. In the present study, we have focused on a novel MT-severing protein called fidgetin, which we posit acts in this fashion. The function of such severing would be to pare back the labile domains of the MTs so that they achieve a proportion relative to the stable domains appropriate for meeting the specific challenges neurons face in various stages in their lives. A reduction of such severing activity would result in an expansion of the labile domains, which we posit would be conducive to phenomena such as neuronal polarization. An increase in such severing activity, on the other hand, would reduce the labile domains, which we posit would be conducive to phenomena such as cessation of axonal growth or pruning back of supernumerary axons. In support of these ideas, we have found that reduction in fidgetin levels by siRNA causes cultured rodent neurons to polarize faster, and produce more processes per cell body and longer axons, together with a notable increase in labile MT mass. In developing *Drosophila* embryos, fidgetin reduction in targeted neurons causes axons to grow past their normal targets rather than retract. Previous studies have shown that katanin and spastin target MT regions that have post-translationally modified tubulins characteristic of stable MTs. Our present studies indicate that fidgetin knockdown results in a dramatic decrease in the ratio of acetylated to total tubulin in the neuron, suggesting that fidgetin targets MT regions that are deficient in acetylated tubulin. In support of this, two different experimental approaches for experimentally increasing the acetylation status of the MTs rendered them less sensitive to the effects of fidgetin reduction. Collectively, these results accentuate the importance of the labile MT component in the axon, which has generally received less attention than the stable component.

P2079**Board Number: B601****Fidgetin inhibition is a novel strategy for altering the balance of stable and labile components of the axonal microtubule array to treat nervous system disease and injury.**A. Matamoros¹, L. Leo², D. Jean¹, T. Austin¹, D. Sharp³, W. Yu⁴, P.W. Baas⁴;¹Neuroscience/Molecular and Cell Biology and Genetics, Drexel University College of Medicine, Philadelphia, PA, ²Neuroscience, Drexel University College of Medicine, Philadelphia, PA, ³Albert Einstein College of Medicine, Bronx, NY, ⁴Department of Neurobiology and Anatomy, Drexel University College of Medicine, Philadelphia, PA

Effective strategies for treating diseases and injuries of the CNS remain elusive. Recent studies have converged on microtubules (MTs) as a powerful therapeutic target. Neurodegenerative diseases called tauopathies involve the dissociation of tau from MTs in the axon. As a result, MT mass is diminished, and this compromises the axon's ability to maintain proper morphology and organelle transport. Treatments able to correct the MT loss would in theory have profound benefits to patients. Drugs that stabilize MTs have produced encouraging results in preclinical studies on tauopathy, with the same drugs also reported to support regeneration after injury to adult CNS axons. Despite these encouraging results, there are persistent questions as to whether this approach appropriately corrects the MT deficiency. MTs in the axon consist of two domains: a stable domain toward the minus end of the MT and a labile domain toward the plus end. These domains are different from one another in their composition, properties and functions. The labile domains comprise a greater component of the total MT array during development, while the stable domains predominate in adult axons. We have suggested that a better strategy for encouraging an injured adult axon to grow would not be to make the MTs more stable, but rather to promote an increase in the levels of the labile component of the MT mass. In the case of tauopathy, our data suggest that it is the labile component that is most vulnerable to degradation, not the stable component. Therefore, we would question whether notable benefit can be achieved by further stabilizing the remaining MT mass. Here we propose a novel approach aimed at increasing the labile component of the MT mass by suppressing a MT-severing protein called fidgetin. Our data suggest that the normal function of this enzyme is to pare back the labile domains of the MTs, in order to regulate the ratio of the stable and labile MT components of the axon at various stages in the life of the neuron. Our hypothesis is that partially suppressing fidgetin will enable the labile component of the MT mass to swell up, which will restore to the tauopathic axon the MT component that had been lost, and which will make the MT array of the injured adult axon more similar to that of a developing axon, and hence more amenable to growth and regeneration. We will present two sets of experiments on primary cultures of rodent neurons. In the first, we show that fidgetin depletion causes an increase in labile MT mass that is conducive to robust axonal regeneration, even on inhibitory molecules that normally suppress CNS regeneration. In the second, we show that tau depletion results in a loss of labile MT mass that is correctable by fidgetin depletion.

P2080**Board Number: B602****Extracellular nicotinamide protects axons from Wallerian degeneration via multiple pathways in cultured DRG neurons.**S. Tokunaga¹, T. Araki¹;¹PNS, National Ctr Neurology and Psychiatry, Tokyo, Japan

Accumulating evidence suggests delaying axonal degeneration as a possible therapeutic strategy for traumatic and neurodegenerative disorders, including stroke, Parkinson's disease and peripheral neuropathy. Recent studies demonstrated that exogenous application of excess nicotinamide adenine dinucleotide (NAD) or its precursor nicotinamide (Nam), the amide form of vitamin B3, have a therapeutic impact on Wallerian degeneration models, but previous reports demonstrated apparently contradictory results: Wang et. al., showed that application of NAD or Nam at the time of neurite transection *in vitro* can delay neurite degeneration concurrently with increased intracellular NAD and ATP level, while others demonstrated that FK866, a potent inhibitor of nicotinamide phosphoribosyltransferase (Nampt) which synthesizes the substrate for NAD biosynthetic enzyme from Nam, can also delay neurite degeneration concurrently with decreased intracellular NAD. Here we show that excess Nam (≥ 3 mM) application-mediated delay of injury-induced neurite degeneration was unaffected by co-application of FK866 or down-regulation of endogenous Nampt. In contrast, FK866-mediated delay of neurite degeneration was counteracted by Nam (≤ 1 mM) dose-dependently but not nicotinic acid (Na), the acid form of vitamin B3, while Na served as NAD precursor in primary DRG neurons. Our results suggest that the Nam may protect axons independently from Nampt activity, presumably by affecting another enzymatic activity sensitive to FK866.

P2081**Board Number: B603****Nerve Growth Factor Induces Mitochondrial Fission Which is Required for Axon Branching.**L. Armijo Weingart¹, R. Sainath¹, G. Gallo¹;¹Shriners Hospitals Pediatric Research Center, Temple University, Philadelphia, PA

During nervous system development, the formation of branches from axons is essential to the formation of complex neuronal circuitry. In sensory neurons, Nerve Growth Factor (NGF) rapidly (30 min) induces branching through activation of phosphoinositide 3-kinase (PI3K). Recently, mitochondria have emerged as major determinants of the sites of axon branching. However, the mechanism of NGF-induced axon branching remains to be fully elucidated. An understanding of how NGF signaling impacts mitochondria behavior and function during axonal branching is the main goal of our current work. To this end, mitochondria were detected in living axons through labeling with Mitotracker dyes. NGF treatment

decreased the length and increased the number of axonal mitochondria after 15 min of acute treatment, indicative of fission. Consistently, live time-lapse imaging of mitochondria following 5 minutes of NGF treatment revealed mitochondria fission. Direct activation of PI3K using a cell permeable peptide in the absence of NGF copied the effects of NGF. Conversely, inhibition of PI3K using LY294002 blocked the effects of NGF on mitochondria. Inhibition of dynamin related protein 1 (DRP1), a required component of mitochondria fission, using mDivi-1 blocked NGF induced axon branching. Live imaging of axons in the presence of NGF revealed that GFP-DRP1 accumulated at sites of mitochondria fission and endogenous DRP1 was detected in axons through immunocytochemistry. Collectively, these observations indicate that NGF induces mitochondrial fission through a DRP1-dependent mechanism and activation of the PI3K pathway, and that fission is required for NGF-induced branching. The NGF induced fission temporally precedes the formation of branches, consistent with the notion that the fission is required to reorganize the axonal mitochondrial array. In future studies we will further address the mechanistic role of mitochondria fission in the generation of axon branches and the role of PI3K signaling in the induction of fission.

P2082

Board Number: B604

Understanding the Axon Initial Segment Diffusion Barrier at the Nanoscopic Level.

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Polarization is a decisive step in neuronal development and once axonal and somatodendritic domains are generated, they persist over the lifetime of an organism. The axon initial segment (AIS), a stretch of 50 – 100 μm in length, bears a specialized cytoskeleton and maintains polarization by restricting the exchange of axonal membrane molecules with the soma. The adaptor protein ankyrinG is essential for the assembly of this diffusion barrier and tethers ion channels and adhesion molecules to the cytoskeleton. However, the structural organization of the AIS and the molecular mechanism, by which protein diffusion is impeded remain unclear. Here, we use novel single molecule localization based superresolution microscopy in primary hippocampal neurons to investigate the molecular architecture of the AIS and to understand the mechanism by which membrane protein diffusion is locally restricted. To do so, we quantify membrane protein diffusion during neuronal polarization in single cells over time by single molecule tracking and correlate these data to the nanoscopic organization of the AIS. We find that the AIS cytoskeleton is organized in a regularly spaced pattern and that coincident with the appearance of this organization during development, the lateral mobility of membrane molecules is locally reduced. Our results clarify the interdependence between cytoskeletal organization and membrane mobility in the axonal plasma membrane.

P2083**Board Number: B605****Mutations in Tau have Differential Effects on the Aggregation and Microtubule Stabilization Properties of its Isoforms.**Y. Mutreja¹, B. Combs², T.C. Gamblin¹;¹Department of Molecular Biosciences, University of Kansas, Lawrence, KS, ²Department of Translational Science and Molecular Medicine, College of Human Medicine, Michigan State University, East Lansing, MI

Tau is a microtubule associated protein found in six different isoforms in neurons. In the diseased state, tau loses its ability to bind and stabilize microtubules and polymerizes to form large aggregates. Various mutations occur in the tau gene leading to a class of neurodegenerative diseases named FTDP-17. These mutations can alter tau's ability to stabilize microtubules and also enhance its ability to aggregate. It has been observed that with many of these mutations, only a subset of isoforms of tau is deposited as aggregates in the patient's brain. Therefore, we hypothesize that these mutations have differential effects on aggregation and microtubule stabilization characteristics of tau in different isoforms. We also sought to determine whether the position of the mutation would differentially affect the underlying biochemical mechanisms of aggregation and microtubule stabilization. We chose to study three different FTDP-17 mutations R5L, P301L and R406W residing respectively in N-terminal, microtubule-binding repeats (MTBR) and C-terminal regions of various isoforms of tau. The extent and kinetics of aggregation induced by arachidonic acid *in vitro* was monitored using laser light scattering, thioflavin S fluorescence and electron microscopy. The aggregation properties of some isoforms were effected more by the mutations than others. Certain isoforms, such as 2N3R and 1N4R were disproportionately affected by mutations in the N-terminal region and the MTBR. The aggregation kinetics of 0N4R were affected more by mutation than the other isoforms. Mutations in the MTBR have a larger effect than N-terminal and C-terminal mutations on the stabilization of microtubules, but also show differences between isoforms for a given mutation. Our results from this study indicates that aggregation and microtubule stabilization of all the isoforms of tau is not affected in same manner and these effects further are dependent on the location of mutation and could provide a biochemical rationale for their different incorporation into aggregates in various diseases in the FTDP-17 class.

P2084**Board Number: B606****Neurite-specific activation of NADPH oxidase in hippocampal neurons overexpressing cellular prion protein or exposed to A β or TNF α .**K.P. Walsh¹, T.B. Kuhn^{1,2}, L.S. Minamide¹, A.E. Shaw¹, S.J. Kane^{3,4}, L.G. Whittington¹, M.D. Zabel³, G.G. Rodney⁵, J.R. Bamburg^{1,4};¹Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO, ²Chemistry and Biochemistry, Univ of Alaska, Fairbanks, AK, ³Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, CO, ⁴Cell and Molecular Biology Program, Colorado State University, Fort Collins, CO, ⁵Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX

Persistent exposure of neurons to soluble β -amyloid peptide dimer/trimers (A β d/t) or proinflammatory cytokines (TNF α , IL-1 β , IL-6) induce the formation of cofilin-actin rods in a subset of neurons (~23%) and in a subset of neurites (~30%) of those neurons. Rods disrupt synaptic function by blocking transport and/or sequestering cofilin from dendritic spines. Rod formation induced by these agents is dependent on the expression of the cellular prion protein (PrP^C) and the subsequent activation of NADPH oxidase (NOX) and production of reactive oxygen species (ROS) (Walsh et al., PLoS One, 9, e95995, 2014). Overexpression of PrP^C-EGFP in the absence of other treatments induces rods in up to 40% of neurons, but the percentage of neurites forming rods does not change significantly. Here we utilized p47^{PHOX}-roGFP (Pal et al., PLoS One 8, e63989, 2013) as a redox probe for measuring NOX-2 activity in living neurons allowing us to localize ROS to the neurites in which it is produced. We generated adenovirus for expressing the probe in neurons and in some experiments also co-infected neurons with adenovirus for expressing cofilin-R21Q-mRFP, a probe for visualizing cofilin-actin rods but one that does not induce rods through its overexpression (Mi et al., PLoS One 8, e83609, 2013). Our results show that NOX-2 activation and ROS production only occur in a subset of neurites from neurons expressing PrP^C-EGFP, even though the PrP^C-EGFP is relatively uniformly distributed among the neurites. Similarly, neurons treated globally with A β d/t or TNF α only show NOX-2 activity, ROS production and rod formation in a subset of neurites. These findings suggest that although PrP^C levels may limit the overall neuronal rod response to A β d/t or TNF α , some factor downstream of PrP^C is the limiting factor for rod formation and this factor is differentially distributed among neurites. The factor is neither cofilin nor actin, since cofilin-actin rods can be induced in almost all neurons and neurites in a PrP^C- and NOX-independent manner by treatment with excitotoxic levels of glutamate. (Supported in part by NIH AG044812 to JRB).

P2085**Board Number: B607****An unbiased genetic screen using the *Drosophila* wing as a model identifies the essential role of a novel gene, *SAD*, in maintaining axonal integrity during aging.**X. Cao¹, Q. Li¹, X. Teng², Y. Zhu², Z. Ma¹, N.M. Bonini², Y. Fang¹;¹SIOC, IRCBC, Chinese Academy of Sciences, Shanghai, China, ²Department of Biology, University of Pennsylvania, Philadelphia, PA

Axon degeneration is a prominent feature of spinal cord injury and many human degenerative diseases. Studies of the Wallerian degeneration slow (*Wld^S*) mouse indicate that axon degeneration is an active process, however, the underlying mechanisms remain elusive. To identify novel molecular components controlling axonal integrity, it is desirable to perform unbiased, large-scale screening. We have developed a novel model of axon injury using the *Drosophila* wing^{1,2,3}. The fly wing is translucent, allowing us to highlight the axons using fluorescent proteins and to monitor axonal changes in response to traumatic injury and aging in live flies.

Using this model, we have performed forward genetic screens to discover novel genes that can protect the injured nerve or revert the *Wld^S*-mediated axonal protection. We found a mutant line of a functionally uncharacterized gene, which not only diminishes the *Wld^S* protection, but also displays striking age-dependent spontaneous axon degeneration on its own. For this phenotype, we named the mutant: *Spontaneous Axon Degeneration (SAD)*. Further examination reveals massive vacuoles in the brain of aged *SAD* flies, indicating progressive neurodegeneration in the CNS as well. Moreover, the lifespan of the *SAD* mutant is significantly shortened. In addition, aged *SAD* flies have elevated sensitivity to stress such as heat and physical disturbance.

Feature analysis of the *SAD* protein suggests that it may be involved in chromatin remodeling and epigenetic modifications. Ongoing experiments include generating *UAS-SAD* and *RNAi-SAD* transgenic flies to determine the role and molecular mechanisms of *SAD* in maintaining neural integrity during aging. And, our most recent behavior assay results point to a non-cell autonomous mechanism of the *SAD* function in the nervous system. All together, by this study, we hope to provide important foundation for new therapeutic targets of axonal/neural degeneration in acute injury as well as chronic neurological disorders.

References:

1. Fang, Y., Soares, L., and Bonini, N.M. (2013) "Design and Implementation of In Vivo Imaging of Neural Injury Responses in the Adult *Drosophila* Wing." *Nature Protocols*, 8(4): 810-819.
2. Fang, Y., and Bonini, N.M. (2012) "Axon Degeneration and Regeneration: Insights from *Drosophila* Models of Nerve Injury." *Annual Review of Cell and Developmental Biology*, 28: 575-597.
3. Fang, Y., Soares, L., Teng, X., Geary, M., and Bonini, N.M. (2012) "A Novel *Drosophila* Model of Nerve Injury Reveals an Essential Role of *Nmnat* in Maintaining Axonal Integrity." *Current Biology*, 22(7): 590-595.

P2086**Board Number: B608****Disruption of Intraneuronal Tau by Extracellular Tau Oligomers Containing Individual or Mixed Tau Isoforms.**E.M. Swanson¹, L. McMahon¹, S. Som², G.S. Bloom²;¹Department of Biology, University of Virginia, Charlottesville, VA, ²Departments of Biology and Cell Biology, University of Virginia, Charlottesville, VA

Tau is microtubule-associated protein enriched in the axons of neurons, where its functions include direct binding and stabilization of microtubules and regulation of axonal transport. Tau is found in the central nervous system (CNS) in six isoforms produced by the alternative splicing of a single tau gene, MAPT, with these isoforms characterized by the presence of zero, one or two N terminal inserts, and three or four C-terminal microtubule binding repeats. Neuronal inclusions composed of hyperphosphorylated tau are a major histopathological feature of a series of neurodegenerative disorders known collectively as tauopathies, the most common and well-known of which is Alzheimer's disease (AD). While the clinical and histological presentation of these disorders is heterogeneous, a majority share the following hallmarks: loss of the normal axonal distribution of tau; accumulation of insoluble, fibrillar tau aggregates in neurites and perikarya; synaptic dysfunction; and eventual neuron death. There is also evidence that the majority of these disorders spread through the brain by a prion-like mechanism, in which pathologically misfolded tau is taken up by neurons, confers this pathological phenotype to the endogenous "normal" protein through direct protein-protein contact, and is eventually released by the cell, perpetuating the cycle. Some evidence points to short tau fibrils as being prion-like, but scant attention has been paid to small tau oligomers. Using a new morphometric method for rigorously quantifying tau distributions in cultured neurons, we now demonstrate that externally applied tau oligomers are much more effective than tau monomers or fibrils at disrupting the normal tau distribution in primary cortical neurons, that oligomer potency varies according to tau isoform, and that oligomers made from mixtures of all six CNS tau isoforms are much more potent than oligomers made from individual isoforms. These results raise the possibility that soluble, pre-fibrillar oligomers of mixed tau isoforms account for the prion-like spread of tau pathology in vivo in AD and non-Alzheimer's tauopathies.

P2087**Board Number: B609****Tau hyperphosphorylation in human Alzheimer's Disease (AD) cases: A quantitative histological comparison.**D. Havas¹, B. Kerschbaumer¹, J. Attems², H. Hutter³, B. Hutter-Paier¹;¹QPS Austria GmbH, Grambach, Austria, ²Institute of Ageing and Health, Newcastle University, Newcastle, UK, ³Institute of Cell Biology, Histology and Embryology, Med Univ Graz, Graz, Austria

Alzheimer's Disease (AD) is characterized by neurofibrillary tangle (NFT) pathology. Yet, the tangle reflects an end stage of paired helical filament aggregations that are built much earlier and are typically found in neurofibrillary threads. Furthermore, they consist of a large array of differently hyperphosphorylated Tau (pTau), whereas several phospho-sites were found to be especially related to AD. However, today's histological knowledge about appearance, quantity and location of PHFs with different phosphorylation and in different brain regions during disease progression is rather poor. To elucidate the quantitative distribution of different pTau sites in the AD brain, we analyzed total human Tau, pThr231 as well as pTyr18 Tau in four different cortical brain regions and the hippocampus of each of five AD subjects from three different Braak stages by immunohistochemistry and compared them to the brains of age-matched non-AD controls. In addition ThioflavinS positive NFTs were counted as well. Further markers, especially pSer262 and pSer202/Thr205 will be included. In spite that Tyr18 Tau phosphorylation was published to be an early AD marker, the quantification in the brain rather revealed the drastic load in cortical regions at end stage. The same late stage increase was observed for ThioflavinS positive NFTs and pThr231 Tau positive somata and neurites. However and most importantly both Tyr18 and Thr231 hyperphosphorylation increase much earlier in the hippocampus and are already significant at Braak stages I/II, while NFT formation lags behind. Our findings indicate a special and early vulnerability of the hippocampal formation to Tau hyperphosphorylation at residues Tyr18 and Thr231, which might contribute to the early break-down of memory function in AD subjects. To note, the downstream tangle formation is a sign of the progressive neurodegeneration but turns out to be rather insensitive for the detection of Tau hyperphosphorylation in early AD.

P2088**Board Number: B610****Induced patient neurons as a potential future model system for Alzheimer's disease.**H. Toresson¹, L. Minthon¹, O. Hansson¹, N. Schultz¹, C. Orbjörn², M. Parmar³, U. Pfisterer³;¹Clinical Memory Research Unit, Dept. of Clinical Sciences, Lund University and Skåne University Hospital, Malmö, Sweden, ²Clinical Memory Research Unit, Dept. of Clinical Sciences, Lund University, Malmö, Sweden, ³Wallenberg Neuroscience Center and Lund Stem Cell Center, Department of Experimental Medical Science, Lund University, Lund, Sweden

The underlying mechanisms of Alzheimer's disease (AD) remain unknown. According to the main hypothesis in the field, the amyloid cascade hypothesis, β -amyloid ($A\beta$) is the causing agent and therefore has been the target for massive drug development efforts. Several drug candidates have been tested in phase III clinical trials but so far none has had any clinical effect. These disappointing results highlight the need for intensified research on the disease in humans. We have launched a cell biological initiative within the Swedish BioFinder Study, a large clinical research programme where dementia patients and cognitively healthy elderly are longitudinally phenotyped by e.g. cognitive tests, cerebrospinal fluid (CSF) biochemistry, amyloid-positron emission tomography and advanced magnetic resonance imaging. Additionally, a skin biopsy is taken to obtain fibroblasts.

We have thus far banked fibroblasts from 34 individuals. Preliminary data show that the most efficient way (time, number of cells and induced neuron (iN) conversion efficiency) was to obtain cells from skin explant cultures in 20% serum followed by expansion in defined serum free medium. 12 out of 12 lines tested could be successfully converted to iN cells by growth factor treatment and transduction of the three transcription factors *Ascl1*, *Brn2* and *Myt1l* (under TET-ON transcriptional control) along with the TET-ON transactivator. Several transduced cells adopted neuronal morphology and after 13 days cells expressed the neuronal markers β III-tubulin and MAP-2. Importantly, iN cells also expressed the AD pathology-associated proteins $A\beta$ and the microtubule-associated protein tau. Isoforms of these proteins used clinically as AD CSF biomarkers ($A\beta$ 1-40, $A\beta$ 1-42, and tau (including phosphorylated tau)) were secreted into the medium in measurable amounts.

The value of iN cells as tools to investigate the underlying disease mechanisms in sporadic AD (SAD) will be assessed by two means. The first is comparing iN cell phenotypes from SAD patients with cells from familial AD (FAD) cases with APP, PSEN1 or PSEN2 mutations. The second, and potentially most powerful method for finding unknown disease mechanisms is to perform an unbiased search for correlations between cellular and clinical phenotypes. Our clinical cell biology approach makes it possible to compare cells from cognitively healthy individuals without clinical signs of AD pathology to healthy or AD cases with a long clinical history of AD pathology. Importantly, this human cell biology approach to AD takes the clinical heterogeneity of dementia diseases into account and thus paves the way for future personalised treatment.

P2089**Board Number: B611** **α -Synuclein Modulates Amyloid- β Induced Ectopic Cell Cycle Re-entry of Neurons.**S.S. Thomas¹, G.S. Bloom²;¹Department of Biology and Neuroscience Graduate Program, University of Virginia, Charlottesville, VA,²Departments of Biology and Cell Biology, University of Virginia, Charlottesville, VA

The defining histopathological features of Alzheimer's disease (AD) are extracellular plaques that comprise amyloid- β (A β) peptides and intraneuronal neurofibrillary tangles made from the neuron-specific, microtubule-associated protein, tau. Normal neurons remain post-mitotic through tight regulation of their cell cycle machinery, but in AD and Parkinson's disease (PD), cell cycle proteins are aberrantly activated, causing the normally quiescent neurons to re-enter the cell cycle. Neurons that re-enter the cell cycle progress at least into S-phase, yet instead of dividing they eventually die. In typical cases of AD, approximately 30% of frontal lobe neurons are lost, and aberrant cell cycle re-entry (CCR) may account for as much as 90% of this neuronal death. Similarly, abnormal activation of cell cycle markers, DNA synthesis, and cell cycle related neuron death have been reported to occur in PD. Despite a large body of evidence demonstrating CCR in AD and PD, many questions regarding the underlying mechanism remain unanswered. Previous work from our lab established that amyloid- β oligomers (A β O) induce ectopic neuronal CCR through a mechanism dependent on tau phosphorylation at Y18, S409 and S416 (Seward, et al. J Cell Sci 126: 1278-1276). Using primary mouse cortical neuron cultures as experimental models, we now report that A β O also promote a tau-dependent increase in endogenous α -synuclein, which accumulates in Lewy bodies in PD and in many cases of AD as well. Furthermore, overexpression of the pathogenic A53T, E46K, or A30P mutations of human α -synuclein induces ectopic neuronal CCR independently of A β O, with the A30P mutation generating the most robust response. In contrast, reduction of endogenous α -synuclein using shRNA ameliorates A β O-induced tau phosphorylation at S409 and S416, and consequently prevents neuronal CCR. Taken together, these data implicate α -synuclein as a novel modulator of ectopic neuronal CCR in AD and PD.

P2090**Board Number: B612****Triple miRNA Shank knockdown shows that Shank-cortactin interactions control actin dynamics in neuronal spines and synapses.**H.D. MacGillavry¹, J.M. Kerr², N.A. Frost³, T.A. Blanpied^{4,5};¹Cell Biology, Utrecht University, Utrecht, Netherlands, ²NINDS, NIH, Bethesda, MD, ³Psychiatry, UCSF School of Medicine, San Francisco, CA, ⁴Department of Physiology, Univ of Maryland Sch of Med,Baltimore, MD, ⁵Program in Neuroscience, Univ of Maryland Sch of Med, Baltimore, MD

Proper functioning of the brain relies on the rapid and efficient transfer of signals across neuronal synapses. However, we have little understanding of the molecular mechanisms that establish and modulate the molecular architecture at the postsynaptic density (PSD) and thereby shape the efficiency of synaptic signal transmission. The family of Shank scaffolding molecules (comprising Shank1, 2 and 3) are core components of the PSD that via multiple protein interaction domains link surface glutamate receptors to other scaffolding molecules within the PSD, as well as to the actin cytoskeleton via diverse actin-binding proteins. However, determining the function of Shank proteins in neurons has been complicated because the different Shank isoforms share a very high degree of sequence and domain homology. Therefore, to control Shank content while preventing potential compensatory effects, we developed a miRNA-based knockdown strategy to reduce the expression simultaneously of all synaptically targeted Shank isoforms in hippocampal neurons. Using this approach, we achieved a strong (>75%) reduction in total Shank protein levels at individual dendritic spines, prompting a ~40% decrease in mushroom spine density. Furthermore, miRNA-based Shank knockdown reduced spine actin levels and increased sensitivity to the actin depolymerizing agent Latrunculin A, an effect restored by re-expression of full-length miRNA-resistant human SHANK2. Interestingly, a SHANK2 mutant lacking the proline-rich cortactin binding motif (SHANK2-dPRO) was unable to rescue these defects, suggesting that Shank-cortactin interactions are required for the maintenance and stability of the spine actin cytoskeleton. Furthermore, Shank knockdown reduced cortactin levels in spines and increased the mobility of spine cortactin as measured by single-molecule tracking PALM, suggesting that Shank proteins recruit and stabilize cortactin at the synapse. Consistent with this model, we found that Shank knockdown significantly reduced spontaneous remodeling of synapse morphology, a phenomenon dependent on actin dynamics, that could not be rescued by the SHANK2-dPRO mutant. We conclude that beyond their role as stable scaffolding molecules within the PSD, Shank proteins are key intermediates between the synapse and the spine interior that, via cortactin, permit the actin cytoskeleton to dynamically regulate synapse morphology and function.

P2091

Board Number: B613

Molecular Mechanisms of Neuroprotection in a Drosophila Model of SCA5.

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Spinocerebellar ataxias (SCAs) encompass a large heterogeneous group of late-onset progressive neurodegenerative disorders that result in uncoordinated motor function. Currently, no effective treatments or therapeutics exist to cure these devastating diseases. To facilitate the understanding and eventual treatment of SCAs, it is imperative to gain molecular insight into the processes that drive neurodegeneration. Our strategy is to use *Drosophila* as a model system to explore SCA5. We have identified a pro-degenerative signaling system that responds to mutations in the neuronal spectrin/ankyrin skeleton and ultimately results in the activation of a cysteine-aspartic protease

(caspase) that is both necessary and sufficient for motoneuron and synaptic degeneration (Keller et al., 2011). When any one of seven independent genes in this pro-degenerative signaling system is deleted the progression of neurodegeneration in *Drosophila* is slowed. The identification of genetic perturbations that can protect against degeneration highlight the importance of these signaling molecules as potential therapeutic targets for treatment of neurodegenerative diseases such as SCA. A preponderance of experimental data suggests that protein quality control mechanisms are intimately involved in disease progression of many SCAs, including SCA5. Preliminary data from my lab indicate that components of the conserved autophagic protein degradation machinery are involved in pro-degenerative signaling and removal of these components can protect against degeneration. This research is focused on elucidating new approaches to suppress neurodegeneration in our *Drosophila* model of SCA5 with the goal of discovering novel targets for future therapeutics.

P2092

Board Number: B614

The adhesion-GPCR CELSR3 promotes axonal fasciculation in the embryonic zebrafish.

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Using the zebrafish retinotectal projection as a model system, we previously showed that GPCR signaling is required for axon guidance during normal development. Retinal ganglion cell (RGC) axons that express a dominant negative G-protein subunit $G\alpha S$ fail to cross the midline and misproject to the ipsilateral tectum. $G\alpha S$ promotes expression of the axon guidance receptors Nrp1a and Nrp1b, helping retinal axons cross the midline, likely via cAMP-dependent signaling. This suggests a mechanism by which advancing growth cones can respond to local signals that upregulate cAMP and thereby ready themselves for the next guidance cue they encounter. However which GPCRs and ligands contribute to this process is still unknown. We identified CELSR3 as a candidate by analyzing GEO datasets to find GPCRs expressed in the right time and place to direct growing axons in the visual system. CELSR3 is a member of the adhesion-GPCR with a characteristic a long n-terminal tail decorated with adhesion motifs including EGF-, leucine-rich, and cadherin repeats as well as Ig domains. In the zebrafish retina, CELSR3 is expressed in amacrine cells as well as RGCs. CELSR3 mutants fail to carry out a optokinetic response (OKR) but have not been analyzed for guidance defects. We assessed CELSR3^{-/-} embryos response to the dark flash behavioral paradigm and examined the trajectory of RGC axons by lipophilic dye labeling. We find that CELSR3^{-/-} retinal axons can cross the midline but are defasciculated (4/30 projections $p=0.03$). This result suggests that CELSR3 may primarily function as an adhesion molecule to maintain axonal bundling during neuronal tract formation.

Establishing and Maintaining Organelle Structure

P2093

Board Number: B616

Role of the endoplasmic reticulum during *Legionella pneumophila* infection.

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Legionella pneumophila is an aerobic bacterium that is the causative agent of Legionnaire's disease. Upon inhalation, the bacteria are internalized by alveolar macrophages, where they replicate. The bacterium's type IV secretion system (T4SS), Dot/Icm, releases over 300 proteins into the host cytosol. These proteins are required for the establishment of the *Legionella* containing vacuole (LCV) and replication. Although it is thought that the LCV acquires membrane by hijacking endoplasmic reticulum (ER) derived vesicles, the exact role of various ER domains during infection is still unknown. Here, we show that *Legionella pneumophila* closely interacts with ER tubules during the early stages of infection. We find that reticulon 4 (Rtn4), a mammalian protein that localizes specifically to the tubular domains of the ER, is mono-ubiquitinated by a family of secreted bacterial effectors, known as SdeA-C. The mono-ubiquitination of Rtn4 leads to the formation of detergent-resistant, immobile Rtn4 aggregates around the LCV. Our live-cell imaging studies suggest that ER tubules form a cage-like structure around the internalized bacterium. Formation of this cage occurs within minutes of infection and may play a role in the establishment of the LCV. Depletion of multiple reticulon families negatively affects *Legionella* intracellular growth in Cos7 cells suggesting that ER tubules might play an important role in the establishment of the LCV. Based on these data, our hypothesis is that the LCV also acquires membrane by interacting with ER tubules rather than solely hijacking ER-derived vesicles. We use genetic manipulation of the *Legionella* genome, gene knockdown, enzymatic profiling of SdeC, and live and high resolution microscopy to test our hypothesis. Our studies will fundamentally change our understanding of how *Legionella pneumophila* establishes a niche to replicate within host cells and will open up new avenues for examining other pathogen-containing vacuoles.

P2094**Board Number: B617****Investigating the genetic pathway driving ER dynamics in *Drosophila melanogaster*.**S.A. Beyeler¹, B.E. Riggs², A.Q. Sims¹, H. Hoffman¹;¹Biology, San Francisco State Univ, San Francisco, CA, ²biology, San Francisco State Univ, San Francisco, CA

The endoplasmic reticulum (ER) is the largest organelle in the cell and recently has been shown to change its shape and localization throughout the cell cycle, experiencing the most dramatic changes during mitosis. It has been suggested that these dynamic changes are the result of the homotypic fusion and fission events of the ER. A prior study has shown that homotypic fusion is mediated by integral membrane GTPase Atlastin (Atl), however the pathway driving these dramatic rearrangements is poorly understood. Here we are investigating the interaction between Atl and the integral membrane ER shaping proteins Reticulon-1 (Rtnl1) and Lunapark (Lnp) in *Drosophila melanogaster*. Our hypothesis is that Atl is regulating the interactions between Rtnl1 and Lnp in a GTP dependent manner mediating homotypic fusions events. To investigate our hypothesis we created 3 different transgenic *Drosophila* lines containing Gal4-UAS mediated RNAi inhibition of Atl, Rtnl1, and Lnp. Each line was driven in the *Drosophila* compound eye and examined for a phenotype. Expression of Atl-RNAi displayed a rough eye phenotype in 70% of the progeny, while expression of Rtnl1 and Lnp did not exhibit a phenotype in the eye. Based on our preliminary findings, we plan to examine Rtnl1-RNAi and Lnp-RNAi in combination with Atl-RNAi and examine the compound eye for either an enhancement or suppression of the rough eye phenotype. These results would indicate that Rtnl1 and Lnp are in the same pathway as Atl, and provide the bases for a modifier screen of the *Drosophila* genome.

P2095**Board Number: B618****Novel ER shaping proteins Pex30 and Pex31 are involved in pre-peroxisome vesicle biogenesis.**A.S. Joshi¹, W. Prinz²;¹NIDDK, NIH, Bethesda, MD, ²NIDDK, National Institutes of Health, Bethesda, MD

The peripheral endoplasmic reticulum (ER) forms a network of flat sheet-like cisternae and highly dynamic interconnected tubules that extend through out the cell. Reticulons and reticulon-like proteins are required to maintain this structure. *S. cerevisiae* has three of these proteins: Rtn1p, Rtn2p, and Yop1p. Interestingly, in cells missing these proteins (*rtn1rtn2yop1delta*) the peripheral ER is mostly converted to sheets. However, a few ER tubules are still found in these cells, suggesting that there may be additional ER tubulating proteins. A novel genetic screen was designed to identify the proteins that tubulate ER in *rtn1rtn2yop1delta*. We found that overexpression of Pex30p and Pex31p restores ER

tubules in *rtn1rtn2yop1delta* suggesting that Pex30 and Pex31 are ER tubulating proteins. Like reticulons and Yop1p, Pex30p and Pex31p are localized mainly in the peripheral ER and absent from nuclear membranes. Unlike reticulons and Yop1p, both Pex30p and Pex31p contain dysferlin domain of unknown function. We show that this domain is required for ER membrane tubulation. Our studies also establish that dysferlin domain is functionally conserved from humans to yeast. In *S. cerevisiae*, Pex30p and Pex31p maintain the number and size of peroxisomes respectively. Mature peroxisomes are derived from pre-existing vesicles known as pre-peroxisomal vesicles (PPV). While the origin of PPV is not known, a recent study show that Pex14p containing PPV are present even in *pex3atg1delta* cells. We investigated whether ER tubulating proteins play a role in PPV biogenesis. We found a significant increase in Pex14p containing vesicles in *rtn1rtn2yop1pex30pex3atg1delta* cells indicating that ER tubulating proteins negatively regulate PPV formation. Taken together, our results suggest that ER shaping proteins are essential for tubulating a subdomain of ER involved in PPV biogenesis. The mechanism of PPV formation remains to be investigated.

P2096

Board Number: B619

Investigating the Role of Myosin 18A in Golgi Morphology.

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Class 18 myosins are closely related to conventional class 2 nonmuscle myosins (NM2) but surprisingly lack intrinsic motor activity. Previous work has shown that M18A binds the Golgi protein GOLPH3 to stretch and shape the Golgi apparatus (Dippold et al., Cell, 2009), although it remains unclear how “motorless” M18A could provide the force necessary to accomplish this task. Recent work from our and Jim Seller’s labs has shown both in vitro and in live cells that M18A co-assembles with NM2 into mixed bipolar filaments. Therefore, M18A may serve to link these hybrid NM2 filaments to the Golgi apparatus, allowing NM2 motor activity to then drive changes in Golgi morphology. The goals of the current study were (i) to use high resolution and super-resolution microscopy to image M18A, NM2, the actin cytoskeleton, and the Golgi apparatus during steady-state and dynamic processes, and (ii) to reexamine the functional role played M18A in determining Golgi shape and size. Using standard Golgi reporters and fluorescently-tagged M18A, we showed that M18A localizes to some extent to the Golgi at steady-state. Additionally, we commonly observed enrichment of actin filaments near the Golgi apparatus. To suppress or eliminate M18A expression, we utilized lentiviral-mediated shRNA knockdown in HeLa cells (HeLa-M18A-KD) and CRISPR-mediated knockout in Rat2 fibroblasts (Rat2-M18A-KO). Surprisingly, we observed no obvious differences in Golgi localization or distribution in KD and KO cells at steady-state relative to control cells. To investigate further, we utilized Brefeldin A (BFA), a small molecule that induces Golgi dispersal, to determine if M18A plays a role during a more dynamic Golgi process. Specifically, we localized M18A and actin during Golgi recovery following removal of BFA. Preliminary results suggest that there is a delay in the recovery of normal Golgi architecture in HeLa-

M18A-KD cells. Moreover, live-cell imaging showed that the localization of M18A and actin on the Golgi apparatus may be enhanced during the recovery process. Overall, therefore, our data suggest that M18A is not important for Golgi morphology at steady-state, but may play a significant role during dynamic Golgi movements, such as those that occur during cell migration or following cell division.

P2097

Board Number: B620

Identification of Rab41 effectors implicated in Golgi organization and trafficking.

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By homology, Rab6a, Rab6a', Rab6b, Rab6c and Rab41 can be grouped together in Rab VI subfamily with Rab6a/a' and Rab41 sharing 80% over much of the protein. Rab6a/a', collectively referred to as Rab6, is the most abundant Golgi Rab protein and localizes to the trans-Golgi cisternae and TGN membranes. Neither depletion nor overexpression of GDP-locked Rab6 results in disruption of the Golgi ribbon and in epistasis experiments, co-depletion of Rab6 suppresses ZW10/RINT1- or COG-dependent Golgi disruption. By electron tomography, Rab6 depletion induces an accumulation of COPI- and clathrin-coated vesicles that is accompanied by an increase in Golgi cisternal number and cisternal continuity. In sum, Rab6 may be more important to Golgi associated membrane trafficking than to Golgi organization. In striking contrast, both Rab41 depletion and overexpression of GDP-locked Rab41 cause Golgi fragmentation into a cluster of punctate elements, suggesting that Rab41 has an active role in maintenance of Golgi ribbon organization. Here, our goal has been to identify the Rab41 effectors producing this phenotypic contrast. To identify how Golgi organization is regulated by Rab41, we screened for Rab41 effectors using the yeast two-hybrid assay. Approximately 5.6×10⁶ yeast transformants were screened from which 265 colonies were isolated under stringent conditions. Following PCR reactions and restriction digestion, 155 non-repetitive hits were isolated and sequenced. 8 putative Rab41 membrane trafficking or vesicle transport effectors were identified. Significantly, none of the 155 hits overlapped with any published Rab6 effector. Syntaxin-8, dynactin subunit 6 and kif18A were selected for further study as candidate effectors of Golgi organization and trafficking. Comparative yeast two-hybrid experiments indicate a preferential interaction of syntaxin-8, dynactin subunit 6 and kif18A with GTP- versus GDP-locked Rab41. Co-immunoprecipitation experiments are in progress to test the interactions of these putative Rab41 effectors with GTP- versus GDP-locked Rab41. More importantly, our studies show that, if dynactin subunit 6 or syntaxin-8, but not kif18A, were depleted using siRNA, the Golgi apparatus displayed a Rab41 knockdown phenotype, i.e., the Golgi apparatus was disrupted into a cluster of punctate Golgi elements. This strongly suggests that, in contrast to Rab6, Rab41 may act directly to regulate Golgi organization through dynactin/dynein recruitment and indirectly through syntaxin-8 dependent membrane trafficking.

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P2098**Board Number: B621****A Trans-Golgi Specific Delay in Cargo Transport Is Sufficient to Explain Increased Golgi Cisternal Number in Rab6-Depleted Cells: A Test of a Delayed Cisternal Progression Model.**L. Dickson¹, S. Liu¹, B. Storrie²;¹Univ Arkansas Med Scis (UAMS), Little Rock, AR, ²Physiology and Biophysics, Univ Arkansas Med Scis (UAMS), Little Rock, AR

Rab6 is the most abundant and studied Golgi-associated Rab protein, and is located predominantly at the exit or trans side of the Golgi apparatus. Knockdown of Rab6 results in a selective dilation of the trans-Golgi network (TGN), an accumulation of trans/TGN proximal COPI- and clathrin-coated vesicles, and an increase in Golgi cisternal number¹. These changes in Golgi organization are accompanied by a delay in cargo transport between the Golgi apparatus and plasma membrane¹. We hypothesized that this phenotype could be the outcome of a trans-Golgi/TGN specific delay in COPI-dependent Golgi recycling and hence a delay in cisternal progression. Here, we tested a key prediction of this model, namely, there should be a trans-Golgi specific delay in cargo transport through the organelle. Taking VSV-G-GFP as a model cargo protein, we determined the kinetics of cargo transport relative to cis, medial, trans and TGN46 markers using confocal fluorescence line scanning. Our results showed that cargo in Control and Rab6-depleted cells moved at a similar rate through the initial Golgi compartments (cis and medial). Cargo exit from both the medial Golgi and TGN occurred with similar rapidity, ~3 min. However and importantly, VSV-G transport between the medial Golgi and TGN was selectively delayed, 30 min versus 10 min for Control. Co-localization experiments with trans Golgi markers GalT and SialylT show that the delay was trans Golgi-specific. Taking the delay in cargo transport as an indicator of delayed cisternal progression, we propose that the observed increase in cisternal number with Rab6 knockdown is the predictable consequence. Experiments that test whether the “inserted” Golgi cisternae are trans-specific by Immunogold labeling are in progress. Results are being further validated by biochemical assays of protein glycosylation.

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1. Brian Storrie, Massimo Micaronii, et al. “Electron tomography reveals Rab6 is essential to the trafficking of trans-Golgi clathrin and COPI-coated vesicles and the maintenance of Golgi cisternal number”. *Traffic* 13(5): 727-44.

P2099**Board Number: B622****Quantitative analysis of Golgi size changes during interphase in mammalian cells.**A. Sin¹, R.E. Harrison¹;¹Department of Biological Sciences, University of Toronto Scarborough, Toronto, ON

During the cell cycle, organelles and genetic materials need to be duplicated to ensure there is sufficient cellular content for daughter cells. As the Golgi complex plays an essential role in the biosynthetic pathway for all living cells, a fundamental question in cell biology is whether Golgi duplication occurs in mammalian cells. Although this process has been studied in parasitic protozoa, yeast, plants and insects, findings from such research may not be directly applicable to mammalian cells due to the substantial differences in Golgi morphology amongst the species. As careful, extensive research has already been performed on Golgi dispersal and inheritance during mitosis in mammalian cells, the aim of our study is to understand the growth of the mammalian Golgi complex prior to mitosis and elucidate the underlying mechanisms that control this process. Through the use of flow cytometry, we have shown that the mammalian Golgi complex has increased structural protein and enzyme contents as the cell cycle progresses, doubling its levels at late G2. Through 3D reconstruction and volumetric analyses after confocal microscopy, it is observed that the cis-, medial- and trans- cisternae concomitantly double in volume at late G2. To investigate the possibility of cell cycle control on Golgi growth, pharmacological agents were used to prolong specific cell cycle phases for 24 and 48 hours. Our results show that synchronized cells had significantly higher Golgi protein levels than those unsynchronized suggesting a lack of checkpoints that prevent indefinite Golgi growth. Interestingly, cell cycle synchronized cells were significantly larger in cell size than those unsynchronized establishing a correlation between Golgi size and cell size. We aim to further investigate the connections between these parameters in our future studies.

P2100**Board Number: B623****Monoubiquitination regulates Golgi membrane dynamics in the cell cycle.**X. Zhang¹, S. Huang¹, Y. Wang¹;¹Dept. of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI

Partitioning of the Golgi membrane into daughter cells during mammalian cell division occurs through a unique disassembly and reassembly process. Several converging lines of evidence have suggested that monoubiquitination plays an essential role in the regulation of post-mitotic Golgi membrane fusion. Monoubiquitination, as a regulatory signal, occurs during mitotic Golgi disassembly and is required for subsequent Golgi reassembly. The AAA ATPase p97 and its adapter protein p47 are involved in membrane fusion during post-mitotic Golgi reassembly. The p97/p47 complex binds to monoubiquitin through the UBA domain of p47, and this interaction is required for p97-mediated Golgi membrane

fusion. Proteasome activity is not involved in either Golgi disassembly or reassembly. We have identified a Golgi-localized ubiquitin E3 ligase HACE1 which is involved in mitotic Golgi disassembly; reduced HACE1 activity in human cancer such as Wilms' tumor results in fragmented Golgi. We have also discovered the p97/p47 binding protein VCIP135 as a deubiquitinating enzyme whose activity is required for post-mitotic Golgi reassembly. VCIP135 activity is regulated by phosphorylation in the cell cycle; it is inactivated by phosphorylation in metaphase and activated upon dephosphorylation in telophase. Recently, we have identified the substrates on the Golgi membranes whose ubiquitination and deubiquitination are regulated by HACE1 and VCIP135 in the cell cycle. These data suggest that cycles of addition and removal of ubiquitin to and from substrates regulate Golgi membrane dynamics during cell division.

P2101

Board Number: B624

Essential role of Golgi-derived microtubules in Golgi positioning and function at the mitotic entry.

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In interphase vertebrate cells, the microtubule (MT) minus end-directed motor dynein localizes to Golgi membranes and organizes them into a single complex in the proximity of the centrosome. This mechanism is thought to facilitate proper partitioning of the Golgi membranes in mitosis, and Golgi complex reassembly in the daughter cells. Yet, the exact mechanism(s) responsible for segregation of the Golgi during cell division remain unclear. It has been proposed that the Golgi membranes split into two groups and move apart with the separating centrosomes during transition from G2 to mitosis. This view has been recently challenged by the finding that dynein dissociates from the Golgi in late G2, breaking the connection to the centrosome. To discriminate these conflicting ideas, we followed the movements of the centrosome and Golgi in human cells via high-resolution multi-dimensional microscopy, and assessed the distribution of the Golgi membranes by a newly-developed computational approach. We found that late in G2, Golgi stacks redistributed independently of the centrosome separation. However, Golgi stacks did not scatter randomly in the cytoplasm, but slid alongside the nuclear envelope approaching even distribution around the nucleus. Furthermore, we detected extensive nucleation of MTs at the Golgi at this cell cycle stage. Specific elimination of Golgi-associated MT nucleation by a number of alternative approaches prevented Golgi redistribution around the nucleus, indicating that Golgi-derived MTs were essential for this process. We also found that Golgi redistribution required recruitment of dynein to the nuclear envelope, which normally occurs in late G2/prophase. Together, our data indicate that redistribution of the Golgi stacks at the G2-mitosis transition is driven by nuclear envelope-localized dynein that acts along the Golgi-derived MTs. A

corollary of this mechanism is that Golgi-derived MTs are responsible for equal segregation of Golgi membranes between the daughter cells. Indeed, we found that Golgi distribution in proliferating cell populations became irregular upon depletion of Golgi-derived MTs. Additionally, we propose that the described mechanism of Golgi redistribution in prophase serves to properly position Golgi-associated membrane coat protein complexes (COPI), which are essential for nuclear envelope breakdown.

P2102

Board Number: B625

Differential Expression, Sorting and Segregation of Golgi Localized Proteins during Germ Cell Differentiation in the Testis.

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Germ cell differentiation in the testis is accompanied by dramatic changes in Golgi apparatus structure and function. These coincide with mitosis of spermatogonia, meiosis of spermatocytes, acrosome formation by early spermatids, and Golgi migration in late spermatids undergoing extensive changes in plasma membrane and cell shape remodeling. Protein based mechanisms are lacking since the protein complement of germ cell Golgi apparatus during these modifications is unknown. To address this, abundant proteins were characterized quantitatively by tandem mass spectrometry of germ cell Golgi fractions isolated from adult rat whole testis homogenates. From 1318 proteins assigned to 22 functional categories, antibodies were generated or obtained to 20 of these proteins. The most abundant was GL54D, a protein of unknown function not previously uncovered but shown here to be a Golgi localized type II integral membrane glycoprotein and germ cell specific. A further protein of unknown function, TM9SF3, was a new Golgi marker for both somatic and germ cells, with 2 other TM9 protein family members predicted as Golgi markers based on extensive organelle sub-fractionation. Besides GL54D and TM9 family members, there were 240 characterized proteins of unknown function enriched in germ cell Golgi fractions. During acrosome formation, different Golgi localized proteins were segregated to Golgi apparatus or were shared with the forming acrosome. The former retained Golgi identity as the Golgi apparatus migrated away from the acrosome after its formation with the latter Golgi proteins remaining trapped in the acrosome. Based on the maturation model for acrosome formation, such segregation is unexpected. During acrosome formation, a subset of Golgi localized molecular chaperone and protein-folding enzymes were uncovered to handle the enormous biosynthetic load. Spermatocytes and spermatids during and after acrosome formation were associated with specific Golgi proteins along with others expressed throughout differentiation. Also unexpected was the finding of a burst and selectivity of expression of Golgi localized proteins to the terminal step 19 spermatid enriched in unstacked flattened cisternae of previously unknown function. When the selective expression and localization of the 20 Golgi localized proteins were compared with 30 proteins characteristic of PM, endosome, ER, coat and cytosol, organelle based molecular signatures for all 63 morphologically distinct germ cells were uncovered. In this way, a marker-based resource with insight

from the cognate protein functions has been established to uncover new mechanisms and proteins in Golgi membrane trafficking and fate during differentiation.

P2103

Board Number: B626

Golgi defects in Alzheimer's disease.

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Golgi fragmentation occurs in neurons of patients with Alzheimer's disease (AD), but the underlying molecular mechanism causing the defects and the subsequent effects on disease development remain unknown. In this study, we examined the Golgi structure in APP^{swe}/PS1 Δ E9 transgenic mouse and tissue culture models. Our results suggest that A β accumulation leads to Golgi fragmentation by activating cdk5, which in turn phosphorylates GRASP65 and perhaps other key proteins critical for maintaining Golgi morphology. Significantly, rescue of Golgi structural defects by inhibiting cdk5 or by expressing nonphosphorylatable mutants of GRASP65 reduces A β secretion by elevating non-amyloidogenic APP cleavage. These results reveal Golgi fragmentation as an important mechanism through which A β may exert its toxic effects. A major potential unrecognized source of A β toxicity may be that it compromises Golgi integrity and perturbs the proper trafficking and processing of many proteins essential for neuronal function. We hypothesize that in AD, A β accumulation promotes Golgi defects, which in turn accelerate APP trafficking and A β production; this deleterious feedback circuit would impair the integrity of the secretory pathway and thereby compromise neuronal cell function. Our study provides a molecular mechanism for Golgi fragmentation and its effects on APP trafficking and processing in AD, suggesting Golgi as a potential drug target for AD treatment.

P2104

Board Number: B627

Blocking iron release alters the behavior of transferrin-containing endosomes in epithelial cells.

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Intracellular iron transport occurs by the binding of two iron molecules to transferrin, which is then internalized upon binding to the transferrin receptor and subsequent clathrin-mediated endocytosis. Upon endosomal acidification, iron is released from the transferrin-transferrin receptor complex and is finally taken up by the mitochondria, which is the principal site of iron-sulfur clusters synthesis and iron incorporation into haeme group. However, the mechanism at the basis of the endosomal iron transfer to mitochondria remains poorly understood. Recently, it has been established that iron can be

transported via a direct interaction (“kiss and run”) between transferrin-containing endosomes and mitochondria. To characterize these “kiss and run” events we have employed time-lapse live-cell TIRF microscopy to simultaneously track fluorescently labeled transferrin-containing endosomes and mitochondria in epithelial cells. Spatio-temporal endosomal parameters such as endosomal speed and distance from mitochondria have been derived using the image analysis software Imaris. We found that a transferrin-containing endosome generally shows significantly reduced speed during its interaction with the mitochondrion (the “kiss” phase) and increased speed during the “run” step of the “kiss and run” event. The role of iron release from transferrin on the behavior of transferrin-containing endosomes and on their ability to interact with mitochondria was investigated using a single point transferrin mutant, which cannot release iron during endocytic acidification. Blocking iron release increased the half-life of the “kiss” phase of transferrin-containing endosome-mitochondria interactions to 416ms compared to 216ms when using wild-type transferrin. Interestingly, we also found that blocking iron release leads to significantly higher endosomal speeds of mutant transferrin containing endosomes (mean speed = 0.58 μ m/s) than that of wild-type transferrin endosomes (mean speed = 0.46 μ m/s). These results suggest that blocking iron release affects endosome-mitochondria interactions resulting in longer interactions with mitochondria as well as increased endosomal speed of transferrin-containing endosomes. In order to address the mechanism underlying the effect of iron release on endosomal speed, we will investigate the role of intra-endosomal milieu such as pH, or extra-endosomal cytoskeletal elements such as actin and tubulin. In the future, we also plan to study the functional relevance of transferrin-containing endosomes and mitochondrial “kiss and run” interactions in cancer cell lines owing to their naturally enhanced iron requirements, altered endocytic pathways, and mitochondrial metabolisms.

P2105

Board Number: B628

The unconventional Myosin Vc is crucial for the transport of cargoes and Rab32/38 from early/recycling endosomes to maturing platelet dense granules in megakaryocytes.

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Mutations of Rab38 in mice and rats result in phenotypes consistent with the mouse models of Hermansky Pudlak Syndrome (HPS), characterized by pigmentation deficiencies and bleeding disorders. HPS is caused by defects in the biogenesis of melanosomes and platelet dense granules, two lysosome related organelles (LROs). Platelet dense granule biogenesis takes place in megakaryocytes and depends upon Rab38 and its close homolog Rab32 for sorting and transport of cargoes to the maturing organelle. We initially identified Myosin Vc (Myo5c) as a protein that directly interacts with Rab32 and Rab38, and characterized its involvement in melanosome biogenesis. Here we show that disruption of the

Rab32/Rab38/Myo5c interaction results in sequestering of Rab32, Rab38 and platelet dense granule cargoes into enlarged early endosomal structures. These structures are marked by Rab5; participate in recycling pathways as demonstrated by co-localization with the transferrin receptor; and appear to be “locked” into a dead-end state as evidenced by fluorescent live-cell microscopy. Characterization of Rab32/38 constitutively active and inactive mutants revealed that the interaction with Myo5c is GTP-dependent. Knockdown of Myo5c function using a *dominant negative (DN)* Myo5c construct consisting of the coiled-coil and globular tail region showed that transport of Rab32 and Rab38 to maturing dense granules is dependent upon wild type Myo5c. Further, Myo5c is absolutely required for transport of cargoes to dense granules as revealed by the failure of known dense granule components (VMAT2, SLC35D3) to co-localize to dense granules in cells transfected with *DN* Myo5c. Last, we investigated the relationship with another Rab32/38 binding partner involved in LRO biogenesis, Varp. Our experiments show that *wild type* Myo5c is anti-correlated with the Rab32/38 effector and endocytic recycling protein Varp; however, knockdown of Myo5c function using *DN* Myo5c results in sequestering of Rab32/Rab38, Varp, dense granule cargoes and *DN* Myo5c at early/recycling endosomes. We propose a handoff mechanism for Rab32/38 marked vesicles from Varp at early endosomes to Myo5c for subsequent transport and delivery to maturing platelet dense granules.

P2106

Board Number: B629

VAMP7 trafficking in melanosome biogenesis: tubular transport into and out of melanosomes.

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Melanosomes are lysosome related organelles (LROs) that coexist in pigmented cells alongside classical endolysosomes. Melanosomal membrane proteins are directed towards maturing melanosomes via membranous transport carriers derived from early endosomal domains, but neither the machinery that mediates fusion of the transport carriers with melanosomes nor a defined pathway for recycling of machinery components to the site of origin for additional rounds of transport are known. To address these unknowns, we focused on VAMP7 as a candidate vSNARE for melanosome transport. VAMP7 has been implicated in melanosome biogenesis because it localizes in wild-type melanocytes primarily to melanosomal membranes at steady state (unlike other endosomal VAMPs), and because depletion of VAMP7 results in mistrafficking of melanosomal cargoes and hypopigmentation. To determine the pathway through which VAMP7 is targeted to melanosomes, we first assessed the subcellular localization of GFP-VAMP7 in melanocytes from mouse models of Hermansky-Pudlak syndrome, in which melanosomal maturation is impaired. Whereas GFP-VAMP7 localized nearly exclusively to pigmented melanosomes in wild type melanocytes, it overlapped nearly completely with syntaxin 13

(STX13) in early endosomes of BLOC-1-deficient cells, analogous to melanosome cargoes like TYRP1. Upon transient rescue of BLOC-1-deficiency, GFP-VAMP7 exited endosomes in mCherry (mCh)-STX13-labeled tubules, and accumulated in nascent STX13-negative structures with features of early stage melanosomes. This suggests that VAMP7 is targeted to melanosomes in STX13-containing endosomal tubules towards maturing melanosomes and is likely the vSNARE on the BLOC-1-dependent cargo transport pathway. To assess VAMP7 recycling from melanosomes, we exploited live cell imaging of tagged VAMP7 in wild-type melanocytes. A second population of tubules labeled by GFP-VAMP7 but not by mCh-STX13 were frequently detected, often emerging from GFP-VAMP7-containing melanosomes. VARP, a scaffolding protein that maintains VAMP7 in an inactive conformation, was largely localized at steady state to puncta that were associated with melanosomes and that were enriched in the tissue-restricted RAB protein, RAB38. By live imaging, tubules labeled by VARP-GFP overlapped with those labeled by mCh-VAMP7 and were frequently detected departing from melanosomes. As expected for recycling structures, these tubules lacked melanosomal cargoes such as TYRP1 and were discrete from the anterograde STX13-positive tubules. These data support a role for VARP in recycling VAMP7 from melanosomes following cargo delivery. Because VARP is a RAB38 effector and overlaps with RAB38 on melanosomal domains, we speculate that RAB38 also functions on this recycling pathway.

P2107

Board Number: B630

A cluster of isolation membrane-associated tubules represents a part of omegasome during final steps of autophagosome formation.

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Recent findings have suggested that autophagic isolation membrane (IM) originates from a domain of endoplasmic reticulum (ER) called “omegasome”. However, its fine structure and detailed positional relationships to the ER and IM during autophagosome formation remain unclear. In the present study, we used *Atg3*-deficient mouse embryonic fibroblasts (MEFs) expressing a marker of omegasome, GFP-tagged double FYVE domain-containing protein 1 (GFP-DFCP1), and found that GFP-DFCP1 was localized on tubular or vesicular elements adjacent to the IM rims by correlative light and electron microscopy and immuno-electron microscopy. Moreover, we developed a fixation protocol for electron microscopy (EM) using a mixture of paraformaldehyde, glutaraldehyde, and osmium tetroxide as a primary fixative, for clear-cut detection of IM and associated vesicular or tubular structures. By EM analyses including serial ultra-thin sections and electron tomography, we observed a cluster of thin tubular structures between the IM edges and ER, part of which were continuous with IM and/or ER. These IM-associated tubular structures (IMATs) were observed in several cell lines and MEFs deficient for *Atg5*, *Atg7*, or *Atg16L1*, but not in *FIP200*-deficient cells, suggesting that they are relevant to earlier events in

autophagosome formation. Taken together, our findings indicate that IMATs represent a part of omegasome during completion steps of autophagosome formation.

P2108

Board Number: B631

Phylogenetic conservation and subcellular localization of apicomplexan SNAREs.

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Apicomplexan parasites are highly polarized eukaryotic cells, with an endomembrane system that includes a minimal ER and Golgi apparatus, and a multitude of distinctive membranous organelles. Dense granules, micronemes and rhoptries are secretory organelles associated with parasite-specific functions, including motility, host cell attachment and invasion, and establishment of a specialized niche for survival within the infected host cell (the parasitophorous vacuole). The Inner Membrane Complex (IMC) consists of a patchwork of Golgi-derived flattened vesicles underlying the parasite plasma membrane and associated with a cytoskeletal scaffold; this assemblage is required for partitioning parasite organelles during cell division. In order to further understand the organellar biogenesis and trafficking, we have identified and partially characterized *Toxoplasma* and *Plasmodium* SNARE proteins (soluble N-ethylmaleimide-sensitive factor adaptor protein receptors). 25 SNAREs are predicted from the *T. gondii* genome, and 24 from the *P. falciparum* genome, and these proteins can be grouped into conventional R/Qabc-SNARE subclasses. Phylogenetic reconstruction, comparison with well-characterized human and fungal SNAREs, and subcellular localization permits identification of clear orthologs for most SNAREs involved in the early secretory pathway (ER/Golgi/TGN trafficking), but not those associated with trafficking to the plasma membrane, lysosomes, etc. Several unusual SNAREs were also identified in the Apicomplexa, including some that localize to the parasite plasma membrane, rhoptries, or uncharacterized cytoplasmic vesicles. This study highlights the divergence of the late secretory pathway in apicomplexan parasites, and provides a map of SNAREs likely to participate in trafficking to both conserved and distinctive organelles.

P2109

Board Number: B632

New Automated Methods to Quantitate Lipid Droplet Number, Fluorescence and Total Volume from Fluorescence Images.

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Lipid droplets are the major organelle for intracellular storage of triglycerides and cholesterol esters. Generally, automated segmentation and quantitation of lipid droplets in fluorescence images of cells has

relied on thresholding-based or watershed methods. We describe here a novel three-stage hybrid method in which candidate lipid droplets are initially identified by thresholding and then tested for circularity. Circular structures are accepted immediately as lipid droplets, while non-circular structures are considered candidate clusters, which are segmented using a watershed algorithm. A unique feature of this method is that the circularity measure can be used as a quality control for the overall segmentation of either stage. The same method can, in principle, be applied with modifications to the estimation of the total lipid droplet volume within a cell. However, as the volume of smaller lipid droplets are difficult to determine accurately by light microscopy, we apply an alternate approach of automatically identifying and fitting the volume of large lipid droplets in calibration images taken parallel to experimental images and identically stained with volume dyes (e.g., Nile Red or Bodipy) and imaged. We describe quantitatively the accuracy of determination of volume of lipid droplets using this method, with respect to factors that must be taken into account, including the degree of reproducibility of staining, the effects of variation in lipid droplet composition and effects of lipid droplet size with respect to several common dyes.

P2110

Board Number: B633

Rapid color changes in the chameleon sand tilefish, *Hoplolatilus chlupaty*.

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The chameleon sand tilefish, *Hoplolatilus chlupaty*, inhabits areas off the coast of Philippines and Okinawa islands in Japan at the depth of 30 to 70m below the surface of the sea. It is well known to hobbyists, scuba divers and zoologists that color of the trunk of the sand tilefish changes from blue to red within less than one second. For this reason, this species also called “flashing tilefish”. In this study, our optical and electron microscopic observations revealed that the coloration and color changes of the chameleon sand tilefish were generated by interference phenomenon of incident light occurred in dermal iridophores. The cytoplasm of the iridophores contained stacked, very thin reflecting platelets, resembling damselfish-type iridophores, but the platelets were arranged and distributed in the limited area nearby the cell membrane. The color changes of the sand tilefish are inordinately fast for fish iridophores, which have been reported to take several seconds or minutes. To investigate the motile activities of the iridophores, we performed pharmacological examinations with scales from trunk of the chameleon sand tilefish. An elevation of K⁺ ions in the perfusing medium gave rise to rapid color changes of the iridophores from blue to red. While acetylcholine did not influence the state of the iridophores, norepinephrine aroused color changes toward reddish color very effectively. The action of norepinephrine on the iridophores was effectively blocked by alpha-adrenergic blocking agent, phentolamine. These results suggest that adrenergic stimulation is a cue to control the motile activities of the iridophores of the chameleon sand tilefish.

P2111**Board Number: B634****The Keap1-Nrf2 redox pathway alters mitochondrial morphology through induced proteasomal degradation of the mitochondrial fission protein Drp1.**T. Shutt¹, M. Geoffrion², R. Screatton³, R. Milne², H.M. McBride⁴;¹Medical Genetics, University of Calgary, Calgary, AB, ²University of Ottawa Heart Institute, Ottawa, ON,³University of Ottawa, Ottawa, ON, ⁴McGill University, Montreal, QC

Mitochondria, best known for their ability to generate both energy and reactive oxygen species also play important roles in many cellular functions. Various signals can affect mitochondrial morphology by altering the balance between fission and fusion events, ultimately influencing metabolic output and sensitivity to cell death. For example, mitochondrial hyperfusion occurs rapidly following several types of cellular stress, in a process described as Stress Induced Mitochondrial Hyperfusion (SIHM). Mitochondria with a hyperfused morphology are refractive to mitochondrial autophagy and provide protection against apoptotic triggers, suggesting that SIHM may play an important protective role during the early stages of cellular stress. This study examines mechanisms regulating mitochondrial shape in response to oxidative stress. We recently demonstrated a role for oxidized glutathione in the direct activation of the mitochondrial fusion machinery, providing a mechanistic link between oxidative stress and fusion. However, questions remain about the persistence of the fused mitochondrial state and whether fission may also be negatively regulated. As part of a genome-wide screen to find novel factors modulating mitochondrial morphology, we identified Keap1. Under normal conditions, Keap1 promotes the ubiquitination and degradation of several target proteins, including the transcription factor Nrf2 (NFE2L2) a key regulator of the cell's antioxidant response. Given that Keap1 is a redox-regulated protein controlling the cellular response to stress and its knockdown results in a hyperfused mitochondrial network, we characterized this novel pathway using a combination of approaches. Although mitochondrial fusion was not activated with Keap1 siRNA, accumulation and nuclear translocation of Nrf2 resulted in a significant decrease in the levels of the mitochondrial fission protein Drp1, explaining the hyperfused mitochondrial network. We then showed that the transcription factor activity of Nrf2 influences mitochondrial morphology by promoting the expression of proteasome proteins and ultimately increasing the degradation of Drp1. Rather than the acute increase in mitochondrial fusion previously described in response to oxidative stress, this novel pathway occurs on a timescale of hours to days to prolong the hyperfused phenotype, thereby protecting the cell from apoptosis until the stress has been neutralized. In summary, we have identified a novel role for the Keap1/Nrf2 redox pathway in maintaining a fused mitochondrial network following cellular stress.

P2112**Board Number: B635****A direct interaction between actin filaments and the dynamin-like GTPase Drp1 supports dual roles for the actin cytoskeleton during mitochondrial fission.**A.L. Hatch¹, H.N. Higgs²;¹Dartmouth College, Hanover, NH, ²Dartmouth-Geisel Sch Med, Hanover, NH

As a dynamic network, mitochondria routinely change morphology in response to different cellular demands and stresses. In this regard, mitochondrial fission and fusion serve several roles: to distribute mitochondria appropriately in dividing or polarized cells; working as a quality control mechanism to help eliminate damaged mitochondrial segments; and even as a component of apoptotic pathways. Defective mitochondrial fission/fusion is found in patients with neurological disorders such as Charcot-Marie-Tooth disease (CMTD), Alzheimer's, Huntington's, and Parkinson's disease. The dynamin-like GTPase Drp1 is a key component of the fission machinery that provides contractile force needed for mitochondrial fission, but a key unanswered question is how Drp1 gets recruited to the mitochondrial outer membrane (OMM). While a number of Drp1 "receptors" have been identified on the OMM, there is also evidence that a mitochondrial pre-constriction step is required prior to Drp1 action. We have shown that actin filaments contribute to this pre-constriction step through the formin INF2, additionally requiring myosin II. INF2 is an endoplasmic reticulum (ER)-bound actin polymerization factor, and mutations in INF2 lead to CMTD. Still, how do actin filaments and pre-constriction lead to Drp1 recruitment? Here, we show that Drp1 binds directly to actin filaments with high affinity. Drp1 binds evenly along the length of the actin filament (shown by electron microscopy), but the interaction is highly dynamic (shown by TIRF microscopy). Actin filament binding increases Drp1's GTP hydrolysis activity, suggesting that actin binding promotes productive Drp1 oligomer assembly. In mammalian cells, Drp1 mutants defective in actin binding do not rescue mitochondrial fission defects induced by endogenous Drp1 suppression. From these results, we propose that actin filaments serve two roles during mitochondrial fission: 1) to provide the force for OMM pre-constriction; and 2) to recruit Drp1 at fission sites through direct binding. In the first role, we propose that actin filaments generate force for pre-constriction with myosin II in a manner similar to cytokinesis. In the second role, actin filaments act as a "co-incidence detector", along with Drp1 receptors such as Mff, Fis1, MiD49, and MiD51. Co-incidence detection of two signals (in this case actin filaments + a Drp1 receptor) is widely used in cells to tightly control activation of important processes. We further propose that other molecules, such as cardiolipin, may serve as independent co-incidence detectors for Drp1 in a similar manner to actin.

P2113**Board Number: B636****Mitochondrial network topology in the absence of fission and fusion dynamics.**M.P. Viana¹, I.A. Mueller¹, C. Goul², S. Rafelski²;¹Developmental and Cell Biology, University of California Irvine, Irvine, CA, ²Developmental and Cell Biology, University California-Irvine, Irvine, CA

Mitochondria form interconnected networks that are constantly reshaped by fission and fusion dynamics. The underlying topological properties of these networks and how fission and fusion together quantitatively contribute to these properties are unclear. We applied an updated version of our MitoGraph method to quantify the topology of mitochondrial networks in budding yeast. We grew cells in microfluidic chambers with constant fresh media in such a way that the cells stopped dividing but continued to grow. This experimental setup permits mitochondrial networks to be at a steady state; because cells are no longer dividing, we avoid effects of the cell being polarized during cell division. We grew cells both in respiring and non-respiring conditions. We found that the number of networks edges and the total mitochondrial content, expressed in terms of the network length, are strongly correlated in both respiring and non-respiring media. This suggests that there is a typical length for edges in mitochondrial networks, similar to what is observed in other types of planar networks. We also investigated the mitochondrial network topology of $\Delta fzo1\Delta dnm1$ yeast cells, which lack the main proteins responsible for mitochondrial fission and fusion. Surprisingly, although networks in these fission/fusion double mutant cells look different by eye, we found no clear topological differences in non-respiring conditions. The only difference we found was that the surface density of mitochondrial tubules was greater in the fission/fusion double mutant cells in respiring conditions and so was their average interconnectivity. To identify the key rules required to generate the topology of mitochondrial networks we simulated many artificial networks generated by different sets of rules and then compared them to the experimentally measured ones. We found two features critical for generating realistic mitochondrial networks both for wild-type and fission/fusion double mutant cells. First, the networks must consist of nodes with only one or three neighbors in a given proportion and second, nodes that are closer to each other in space must be preferentially connected. We conclude that cells in the absence of fission and fusion still generate mitochondrial networks with very similar basic topological properties to wild-type cells, suggesting that there may be processes beyond fission and fusion that determine how mitochondrial networks are generated in yeast cells. We are now using more sophisticated measurements to determine how the visual differences between wild-type and double mutant cells arise.

P2114**Board Number: B637****High-Throughput drug discovery of novel inhibitors of secretion.**A. Satoh¹, H. Suzuki¹, Y. Honjo², Y. Nishina¹;¹Okayama Univ, Okayama, Japan, ²Hiroshima University, Hiroshima, Japan

Finding new candidate molecules with medicinal properties normally depends on serendipity or investment of time and resources. For example, massive chemical library screenings have successfully identified several new drug candidates. To increase the chances of finding such candidates, we used pooled substrates as starting materials. The pools of substrates, respectively consisting of 11 chemicals and 1 catalyst, underwent multiple chemical reactions, resulting in pools of reactants. These pools were tested in cell-based biological assays for cytotoxicity and secretion. One of the pools was not toxic but exhibited secretion inhibition; therefore, we isolated a compound from the pool showing inhibitory activity. As expected, the compound, termed bis-indole 45, disrupted the Golgi without affecting microtubules. Bis-indole 45 was then compared with known secretion inhibitors, Brefeldin A (BFA) and Golgicide A (GCA), in the following tests. BFA and GCA inhibit secretion by inhibiting the function of an Arf-GEF, GBF1, upon freezing Arf and GBF1 on membranes. Bis-indole 45, however, did not exhibit such an effect. GBF1 recruitment to the Golgi membrane is thought to be mediated by the golgin tether p115 and the small GTPase Rab1. Exogenous expression of a dominant negative mutant of Rab1 but not p115 disrupted GBF1 localization to the Golgi in a manner similar to bis-indole 45. These results suggest that bis-indole 45 inhibits secretion by inhibiting GBF1 recruitment to the Golgi through Rab1. As exemplified in this study, drug screening using pooled substrates can be a powerful and useful method for high-throughput drug discovery.

P2115**Board Number: B638****Quantifying Cell Membrane Morphology Changes with a Biophysical Membrane Model.**R. Tourdot¹, R. Bradley¹, R. Natesan², R. Radhakrishnan²;¹Chemical and Biomolecular Engineering, University of Pennsylvania, Philadelphia, PA, ²Bioengineering, University of Pennsylvania, Philadelphia, PA

Organelle shapes are defining characteristics of different types of cells and can help regulate their function, with endosomes and the Golgi apparatus being examples of biological topologies with tightly controlled shape and curvature. A special subset of membrane-recruited proteins has recently been shown to sense and/or induce curvature in the bilayer: such proteins include BAR domain containing proteins and the ENTH domain containing proteins. Emerging research has revealed that these curvature-inducing proteins play a role in several critical cell processes ranging from their incorporation into endocytic pits, and localization to sites of invadopodia formation *in vivo*. Mathematical models relating membrane-protein interaction to membrane morphology can provide physical insight into the

driving forces governing curvature induction and morphological changes in the cell membrane. These models of cell membrane elasticity have been used previously to describe the biconcave shape of red blood cells, and large-scale morphological changes of the cell membrane such as cell division. Our work focuses on applying a sub-cellular (mesoscale) model based on the Helfrich formalism for cell membranes to a curvature inducing protein system, and utilizing it in concert with a suite of computational free energy methods to define the free energy landscape of cell-membrane morphology changes. In particular, our model provides novel insight into the critical density of proteins needed for membrane tubulation in invadopodia formation, and the interplay between local tension and curvature generation in vesicle nucleation in endocytosis. Our research finds that protrusions are considerably longer and more stable under low tensions, and these results corroborate recent experimental results comparing cells cultured in 2D and 3D media, where cells in 3D matrices are found to have lower tension and more prominent cell protrusions.

P2116

Board Number: B639

Understanding the factors regulating size and shape of dynamic organelle: an holistic approach.

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Size and shape control mechanisms of dynamic organelles are of fundamental importance in basic cell biology. More over several such organelles are known to alter their size and shapes during normal physiological processes as well in diseases especially in cancerous cells and this underlies the importance of the subject. We are presently studying the Golgi apparatus and Nucleus size and shape control mechanism using a combination of cell biology and mathematical modeling. Recently we have shown that the size control mechanism of Golgi apparatus possibly being regulated by altering the cisternal maturation kinetics by ARF1 in budding yeast. Using a genome wide screening approach we have identified several other factors affecting Golgi size. Combining advanced imaging techniques with mathematical simulation based methods we have identified few potential mechanisms through which such regulatory mechanisms may operate. Our results indicate that although size and shape control mechanism may differ for different organelles however there are definitive possibilities of mechanistic similarities in such processes.

P2117**Board Number: B640****Nucleolar size scaling through *C. elegans* growth and development.**S. Uppaluri¹, S.C. Weber¹, C.P. Brangwynne¹;¹Chemical and Biological Engineering, Princeton University, Princeton, NJ

The nucleolus is a non-membrane bound organelle that is the site of ribosome biogenesis and is therefore strongly linked to protein translation and cell growth. Requirements for protein translation vary greatly during *C. elegans* development - from a single-celled embryo through four larval stages to adulthood. How nucleolar size and function varies in the context of multicellular growth remains poorly understood.

We have shown that in the early reductive embryonic cell divisions, nucleoli scale with cell size. Upon hatching and through subsequent larval development we find again that nucleolar size scales with cell size. Altering growth rate, as well as ultimate cell size, through environmental and genetic perturbations leads to the same 'universal' scaling. To elucidate the biophysical mechanism governing nucleolar size, we utilize a custom microfluidic platform to instantaneously change cell volume and nucleolar component concentration. Consistent with a simple phase transition model we find that increasing organelle component concentration leads to larger nucleoli. These results point to a cell-size sensing mechanism through which organelle size is regulated.

P2118**Board Number: B641****A dynamin-like ER-shaping protein and FIT proteins regulate lipid droplet biogenesis in yeast.**N. Ojha¹, V. Choudhary¹, W. Prinz¹;¹NIDDK, National Institutes of Health, Bethesda, MD

Lipid droplets (LDs) are ubiquitous neutral lipid storage organelles. LD biogenesis occurs in the ER but is not well understood. FITs are a conserved family of proteins that have been implicated in LD biogenesis. Yeast has two FIT proteins, called Scs3p and Yft2p. We found that a mutant lacking Scs3p and the dynamin-like GTPase Sey1p have a dramatic growth defect. Sey1p is an orthologue of atlastins and facilitates ER-ER fusion. Cells missing Scs3p and Sey1p were found to have a significant increase in the number of lipid droplets and at least a two-fold increase in neutral lipids, suggesting that these cells may have altered LD biogenesis. Remarkably, we found that LDs in these cells were wrapped by a membrane. We ruled out that these membranes are derived from autophagy but instead are part of the ER. LDs in cells missing Scs3p and Sey1p appear to bud towards the lumen of the ER instead of into the cytosol, which may account for the aberrant membrane wrapping. Together, our findings suggest that Sey1p and Scs3p proteins regulate LD number and LD biogenesis, perhaps by facilitating LD budding from the ER.

P2119**Board Number: B642****Analysis of mitochondrial proteins and their effect on mitochondrial dynamics in *Dictyostelium discoideum*.**L. Woods¹, B.G. Schimmel¹, G.W. Berbusse¹, K. Naylor¹;¹Biology, University of Central Arkansas, Conway, AR

Mitochondrial morphology is maintained by two distinct membrane events -fission and fusion. Altering these conserved processes can disrupt mitochondrial morphology and distribution, thereby disrupting the organelle's functionality and impeding cellular function. In higher eukaryotes, these processes are mediated by a family of dynamin-related proteins (DRP's). In the lower eukaryotes, for instance *Dictyostelium discoideum*, mitochondrial fission and fusion have been implicated but not yet established. To understand the overall mechanism of these dynamics across organisms, we developed an assay to identify fission and fusion events and measure organelle velocity in *Dictyostelium* and to assess the involvement of the mitochondrial proteins, CluA, and two DRPs, DymA and DymB. Using laser scanning confocal microscopy to observe real time movement of fluorescently labeled *Dictyostelium* mitochondria, we show that lower eukaryotes mediate mitochondrial fission and fusion. In *Dictyostelium*, these processes are balanced, occurring approximately 1 event/minute. Quantification of the rates of fission and fusion in *cluA*-, *dymA*-, or *dymB*- strains established that the DRP's are not essential for these processes. Rates of fission and fusion were significantly reduced in *cluA*- cells, indicating that CluA is necessary for maintaining both fission and fusion. Interestingly, loss of DymA significantly increases the velocity of organelle movement within the cell though it does not alter the number of mitochondria moving; while loss of CluA has no effect on velocity, it instead decreases the number of organelles that are motile.

We have successfully demonstrated that *Dictyostelium* mitochondria undergo the dynamic processes of fission and fusion. The classical mediators of membrane dynamics, the DRP's, are not necessary for these dynamics though DymA does affect motility, whereas CluA is necessary for both processes and decreases the percentage of motile organelles. This work contributes to our overall understanding of mitochondrial dynamics and ultimately will provide additional insight into the evolution of these processes.

P2120**Board Number: B643****VAMP7 is a biogenesis of lysosome related organelles complex-1 (BLOC-1) cargo required for pigmentation in melanocytes.**A. Acosta-Ruiz¹, M.K. Dennis¹, R.A. Linares¹, C. Delevoye², G. Raposo², M.S. Marks¹;¹Pathology and Laboratory Medicine, University of Pennsylvania Children's Hospital of Philadelphia, Philadelphia, PA, ²Institut Curie, Paris, France

Melanosomes are lysosome related organelles (LROs) within which melanins are synthesized and stored in pigmented cells. Resident proteins are delivered to nascent melanosomes from early endosomes via membranous transport intermediates in a process that requires Biogenesis of Lysosome Related Organelles Complex-1 (BLOC-1) - a protein complex comprised of subunits that are absent in a subset of patients with Hermansky-Pudlak syndrome. The machinery that mediates fusion of these intermediates with maturing melanosomes is not known. As potential fusion machinery components, we focused on VAMP7 and syntaxin13 (STX13), because depletion of either of these SNARE proteins results in hypopigmentation. In normal pigmented melanocytes at steady state, STX13 localizes to early endosomes and tubules that contact maturing pigmented melanosomes, and VAMP7 localizes primarily to the melanosomes themselves. We thus initially proposed a model wherein BLOC-1-dependent, STX13-positive, endosomal carriers fuse with VAMP7-positive early stage melanosomes. We first found that in BLOC-1-deficient pallid melanocytes, STX13-positive endosomal tubules fail to form, consistent with a critical role for STX13-positive endosomal carriers in protein delivery to melanosomes. However, in these cells a GFP-VAMP7 fusion protein was completely retained within mCherry-STX13-positive endosomes rather than in early stage melanosomes as predicted by our initial model; following "rescue" with myc-tagged Pallidin, GFP-VAMP7 localization was restored to newly pigmented melanosomes that segregated from mCherry-STX13-labeled endosomes. These data suggest that VAMP7 is in fact a cargo of the BLOC-1 pathway and of the STX13-containing transport intermediates. To further test this model, we utilized a novel assay to visualize early events in melanosome biogenesis without the confounding effects of predominant steady state localization to mature melanosomes. We transiently rescued BLOC-1 expression in pallid melanocytes by transfection with myc-tagged Pallidin and monitored changes in the localization of coexpressed GFP-VAMP7 and mCherry-STX13 at various time points following transfection. As predicted by our model, the number of GFP-VAMP7-containing structures that segregated from mCherry-STX13-labeled structures increased over time. Moreover, at intermediate time points following transfection, GFP-VAMP7 could be observed by live cell imaging to traffic within mCherry-labeled tubules towards nascent VAMP7+ puncta. These data support a model wherein BLOC-1-dependent endosomal carriers that contain STX13 and VAMP7 fuse with early stage melanosomes in a process required for pigmentation. We propose that VAMP7 functions as the vSNARE for this fusion event.

P2121**Board Number: B644****ENDOPHILIN RAPIDLY BENDS MEMBRANES TO PROMOTE ENDOCYTOSIS.**K.R. Poudel¹, J. Bai¹;¹Fred Hutchinson Cancer Research Center, Seattle, WA

Synaptic Vesicle (SV) endocytosis is responsible for generating synaptic vesicles at the presynaptic terminals. To sustain high-frequency brain activities, endocytosis must act rapidly to regenerate SVs, which prevents vesicles from depletion. Endophilin is a synaptic protein that plays critical role in promoting SV endocytosis. Endophilin contains a Bin–Amphiphysin–Rvs (BAR) domain that has been shown to bend flat membranes into highly curved tubules in vitro. Our recent findings demonstrate that the membrane-bending activity is required for endophilin's function in vivo. Here, we perform biophysical analyses to investigate molecular mechanisms for endophilin to generate membrane curvature. Our preliminary results show that the endophilin BAR domain is an active membrane bender instead of a passive sensor. These results will help us to understand how endophilin acts to support SV endocytosis in neurons.

P2122**Board Number: B645****Three dimensional architecture of Extended-Synaptotagmin-mediated ER-plasma membrane contact sites.**R. Fernandez-Busnadiego¹, Y. Saheki¹, P. De Camilli¹;¹Department of Cell Biology, Howard Hughes Medical Institute, Program in Cellular Neuroscience, Neurodegeneration and Repair, Yale University School of Medicine, New Haven, CT

Growing evidence suggests that the close apposition between the endoplasmic reticulum (ER) and the plasma membrane (PM) plays important roles in Ca²⁺ homeostasis, signaling and lipid metabolism. The Extended Synaptotagmins (E-Syts/Tricalbins in yeast) are ER-anchored proteins that mediate the tethering of the ER to the PM. E-Syt cytoplasmic domains comprise an SMP domain followed by five C2 domains in the case of E-Syt1 and three C2 domains for E-Syt2/3. Here, we used cryo-electron tomography to study the 3D architecture of E-Syts-mediated ER-PM contacts at molecular resolution. We show that, in vitrified frozen-hydrated mammalian cells overexpressing individual E-Syts, ER-PM distance (19-22 nm) correlates with the amino acid length of the cytosolic region of E-Syts (i.e. the number of C2 domains). We further show that the levels of cytosolic Ca²⁺ regulate ER-PM distance at E-Syt1-dependent contacts sites. E-Syt-mediated contacts displayed a characteristic electron-dense layer between the ER and plasma membranes. In contrast, at STIM1-Orai1-mediated contacts the gap between ER and PM was spanned by filamentous structures perpendicular to the membranes. These two types of ER-PM contacts were also observed in the native context of untransfected neurons. Thus, our results define specific ultrastructural features of E-Syts-dependent ER-PM contacts and reveal their

structural plasticity, which may impact on specific E-Syt functions and the cross-talk between the ER and the PM.

Mitochondria 2

P2123

Board Number: B646

Regulated by mitochondrial fusion dynamics, lipid droplets serve as central fatty acid conduit to supply mitochondria with fatty acids for oxidation during nutrient stress.

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Cells must adapt their metabolism to changing nutrient and environmental conditions in order to survive. One key adaptation occurs during nutrient deprivation when cells reprogram their metabolism from glucose to mitochondrial fatty acid oxidation for maintaining cellular energy levels. Although it is well known that endogenous fatty acids act as major energy substrate during nutrient stress, from where and how such fatty acids are mobilized and selectively trafficked to mitochondria without causing damage to membranes and acting as toxic bioactive lipids is a major question. Using a novel imaging based approach to visualize fatty acid trafficking in live cells in combination with metabolic profiling experiments, we revealed that lipid droplets, specialized lipid storage organelles, serve as selective conduit to direct fatty acids into mitochondria for oxidation. We found that fatty acid recycling by bulk autophagy of cellular membranes helped replenish lipid droplets with fatty acids, increasing lipid droplet number over time. From lipid droplets, fatty acids were subsequently liberated by cytoplasmic lipases, and efficiently transferred into mitochondria. Surprisingly, we found that this transfer step required mitochondria to both be localized near lipid droplets and highly fused. Mitochondrial fusion increased the connectivity to lipid droplets and promoted mitochondrial fatty acid distribution and oxidation. When mitochondrial fusion was prevented in cells lacking the major fusion proteins mitofusin 1 or optic atrophy protein 1, fatty acids neither homogeneously distributed within mitochondria nor became efficiently metabolized. Instead, these morphodynamic defects caused massive alterations in cellular fatty acid routing. Not only were non-metabolized fatty acids redirected to and stored in lipid droplets, they became excessively expelled from cells. Together this study highlights the importance of coordinated organelle dynamics to direct cellular fatty acid flow and oxidation during nutrient stress. Given that fatty acids and their derivatives play important roles in regulating cellular signaling cascades in metabolism and inflammatory responses, future work studying the multi-organelle controlled fatty acid flux system described here might be of high potential for contributing to our understanding of the pathophysiology of lipid-associated diseases, such as obesity, diabetes, cancer and inflammatory disorders.

P2124**Board Number: B647****Mastoparan causes mitochondrial permeability transition not by interacting with specific proteins, but by interacting with the phospholipid phase.**T. Yamamoto¹, M. Ito^{1,2}, K. Kuwahara^{1,2}, K. Kageyama^{1,2}, K. Yamashita³, Y. Takiguchi², H. Terada⁴, Y. Shinohara^{5,6};

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Mitochondrial permeability transition causes the release of various proteins from mitochondria, and the released proteins trigger subsequent steps of apoptosis and necrosis. Permeability transition is believed to result from the formation of a proteinaceous pore, but the molecular mechanisms have not yet been revealed. The mastoparan peptide is a tetradeca-peptide isolated from the venom of wasp. Mastoparan is known as an inducer of the mitochondrial permeability transition. Although mastoparan was suggested to interact with a proteinaceous target in mitochondria to induce this transition, the action sites of mastoparan have not yet been investigated. To clarify whether specific interactions of mastoparan with receptors or enzymes are associated with the induction of this permeability transition, we here examined the effects of D-isomeric peptides, which were synthesized by using D-amino acids assembled in endogenous (inverso mastoparan) and reverse (retro-inverso mastoparan) orientations. When we added inverso mastoparan to isolated mitochondria, the peptide caused the permeability transition in a partially cyclosporin A-sensitive manner at lower doses and in a cyclosporin A-insensitive manner at higher ones. The action manners and the potencies of inverso mastoparan were close to those of parent mastoparan, indicating that the targets of mastoparan for induction of the permeability transition were neither receptors nor enzymes in the mitochondria. Retro-inverso mastoparan also had the same effect on the mitochondria as mastoparan, but the potencies of the effect were weaker. Not only on mitochondria but also on phospholipid vesicles, mastoparan and inverso mastoparan showed massive permeabilization effects at the same potencies, but retro-inverso mastoparan showed weaker ones. These results indicate that mastoparan interacted with the phospholipid phase of the mitochondrial membrane, not with specific proteins, to induce the permeabilization in cyclosporin A-sensitive manner and -insensitive manners.

P2125**Board Number: B648****Lipid-mediated interactions of the mitochondrial presequence translocase.**K. Malhotra¹, N. Alder¹;¹Molecular and Cell Biology, University of Connecticut, Storrs, CT

Following the original endosymbiotic event, nearly all of the genes that encode mitochondria-resident proteins have been transferred to nuclear DNA. As a result, the biogenesis of mitochondria is dependent on the efficient import of nuclear-encoded proteins to its different sub-organellar compartments, carried out by the protein translocases present in its two membranes. The inner membrane of mitochondria is a highly protein-rich biomembrane, where the lipid molecules are known to modulate protein structure and function, hence affecting mitochondrial processes. This is probably best exemplified by cardiolipin, the signature phospholipid of mitochondria, which regulates numerous mitochondrial functions, from ATP production to cell death. The Translocase of the Inner Mitochondrial Membrane 23 (TIM23) mediates both the transport of nuclear-encoded precursor proteins across the inner membrane and the lateral integration of membrane proteins. The initial steps during precursor protein transport are largely mediated by the interaction between the central component of the TIM23 Complex, Tim23, and its receptor partner, Tim50. The lipid dependence of the functional interactions of subunits within this translocase have largely remained unknown. Using the Tim23 channel reconstituted into soluble nanoscale bilayers (nanodiscs) and site-specific chemical crosslinking, we show for the first time that the interaction between the co-receptor Tim50 and the channel region of Tim23 protein, is mediated by cardiolipin. To confirm the cardiolipin-dependent interactions of the mitochondrial preprotein translocase seen in this model-membrane system, we employ a host of cardiolipin variant yeast strains, to study interactions between Tim23 and Tim50 within active mitochondria. Further, by performing liposome binding studies combined with Molecular Dynamics simulations, we show that the soluble domain of Tim50 interacts specifically with cardiolipin containing membranes, and specific residues in the β -hairpin loop are required for mediating the interaction. Moreover, our study suggests that the interaction between Tim50^{IMS} and cardiolipin is accompanied by secondary structural changes in the soluble domain of the co-receptor. By allowing the study of membrane proteins using solution-based biophysical techniques combined with *in organello* and *in silico* work, this system offers unprecedented insights into the early stages of Tim23-mediated protein import.

P2126**Board Number: B649****Proper inheritance of mitochondrial content is maintained in budding yeast cells with mitochondrial dysfunctions.**I.A. Mueller¹, M.P. Viana¹, S. Rafelski²;¹Developmental and Cell Biology, University of California Irvine, Irvine, CA, ²Developmental and Cell Biology, University California-Irvine, Irvine, CA

The inheritance of mitochondria from mother to bud is asymmetric and highly regulated in budding yeast, *Saccharomyces cerevisiae*. The mitochondrial-volume-to-cell-volume ratio (mitochondrial volume ratio) is higher in buds compared to mothers at the time of division and is normally maintained independent of the mother's mitochondrial content. Mitochondrial inheritance depends not only on the redistribution of mitochondria between mother and bud but also on mitochondrial biogenesis. Mitochondrial biogenesis requires mitochondrial membrane potential, an indicator of mitochondrial functionality, for protein import into the organelle. Consequently, mitochondrial function should play a pivotal part in proper mitochondrial inheritance, but the exact role of mitochondrial function in mitochondrial inheritance has yet to be investigated. We sought to determine whether mitochondrial dysfunctions of different severities impact mitochondrial inheritance in budding yeast. Mitochondrial dysfunctions were introduced by deletion of cytochrome c oxidase subunit 6 (Δ COX6), inducing loss of respiratory function, or by deletion of mitochondrial DNA polymerase MIP1 (Δ MIP), resulting in loss of respiratory function and ultrastructural alterations. Live-cell, 3D light microscopy images of fluorescently labeled mitochondria of wild type, Δ COX6 and Δ MIP cells were analyzed using our quantitative imaging analysis (MitoGraph) approach to determine mitochondrial tubular width and content at the time of division. Visually, the mitochondrial network morphology in Δ COX6 cells is "wild type-like", while the mitochondrial network of Δ MIP cells is characterized by regionally collapsed and clustered tubules. Mitochondrial tubules are 3% narrower in Δ COX6 cells and 7% narrower in Δ MIP cells compared to wild types ones, suggesting ultrastructural alterations occur in both deletion mutants but are more pronounced in Δ MIP cells. Mitochondrial volume ratio is higher in Δ COX6 and Δ MIP buds compared to their mothers, and more importantly the mitochondrial volume ratio of mutant buds is maintained compared to wild type buds at the time of division. This suggests that asymmetric, actively regulated mitochondrial inheritance is not affected in cells with loss of mitochondrial respiratory function and ultrastructural alterations. Interestingly, the total mitochondrial volume ratio of Δ COX6 cells (mother and bud) is lower compared to wild type cells despite the mitochondrial volume ratio of the bud being maintained, implying problems in mitochondrial biogenesis in Δ COX6 cells. Further studies will determine why total mitochondrial volume ratio is reduced in Δ COX6 but not in Δ MIP cells, even though Δ MIP cells should have more severe alterations in organelle functionality and thus biogenesis.

P2127**Board Number: B650****Extracts from *Alpinia galanga* mediate mitochondria dynamics.**C. Lo¹, C. Chiu², C. Chang²;¹Department of Food Science, National Chiayi University, Chiayi, Taiwan, ²The Institute of Biotechnology, National Tsing Hua University, HsinChu, Taiwan

Mitochondria are highly dynamic organelles with continuous fission and fusion. The dynamics of mitochondria is linked to mitochondrial function and cell metabolism. More and more evidence has indicated that mitochondria dynamics respond to different environmental stress. *Alpinia galanga* has been used as a food spice as well as folk medicine. Curcuminoids purified from *A. galanga* were known to have cytotoxic effects on human leukemic cells and may induce apoptosis through a combination of mitochondrial and endoplasmic reticulum stress pathways. Thus, we aim to clarify whether the extracts from *A. galanga* affect mitochondrial dynamics. Our results demonstrated that treating cervical cancer cells with curcuminoids from *A. galanga* changed mitochondrial morphology and function. These results suggest that curcuminoids from *A. galanga* can be used to further dissect the regulatory pathways of mitochondria dynamics.

P2128**Board Number: B651****Ordered partitioning and constant replication ensure mitochondrial DNA maintenance.**R. Jajoo¹, M.P. Viana², S. Rafelski³, M. Springer¹, J. Paulsson¹;¹Department of Systems Biology, Harvard Medical School, Boston, MA, ²Developmental and Cell Biology, University of California Irvine, Irvine, CA, ³Developmental and Cell Biology, University of California-Irvine, Irvine, CA

Mitochondrial DNA (mtDNA) is located in the mitochondrial matrix and is essential for normal eukaryotic cell function. Like nuclear DNA, mtDNA cannot be created *de novo* and must be accurately replicated and partitioned at each cell division. Unlike nuclear DNA, mtDNA has an endosymbiotic origin, varies in copy number, and does not encode its own replication machinery, complicating its maintenance. Here we uncover the partitioning and replication strategies of mtDNA in the fission yeast *Schizosaccharomyces pombe* by counting mtDNA clusters (nucleoids) in single dividing cells. We find that nucleoids are segregated with half the error expected by “random” binomial partitioning, ensuring that most cells have similar numbers of mitochondrial nucleoids and that mtDNA is rarely lost by any cell. By measuring nucleoid partitioning in mutants that divide asymmetrically, we find that this accuracy is achieved by evenly distributing mitochondria throughout the cytoplasmic volume. Finally, by counting nucleoids at the beginning and end of the cell cycle, we show that the nucleoid replication rate is independent of the number of nucleoids present. Therefore, it appears that mitochondrial nucleoids

avoid the instability inherent in self-replication by ceding replication control to the host cell. In total, mtDNA avoids being lost through a combination of ordered partitioning and constant replication.

P2129

Board Number: B652

Novel mitochondria-microtubule crosstalk regulates mitochondrial biogenesis and morphology.

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Mitochondrial homeostasis requires a complex coordination of biogenesis, organization, function, dynamics, and inheritance. Here we identify novel regulation of mitochondrial homeostasis mediated by cross talk with tubulin proteins. We previously conducted a genome wide screen to identify genetic interactions for mutants of yeast α - and β -tubulins that lack negatively charged carboxy-terminal tail regions (CTTs), which project from the microtubule surface. We uncovered a number of mitochondrial genes, including genes involved in biogenesis, morphology, and inheritance. This suggested a role for tubulin CTTs in regulating mitochondrial homeostasis; especially surprising because the actin network in yeast drives mitochondrial transport. To test the hypothesis that tubulin CTTs are important for mitochondrial homeostasis, we imaged live mutant yeast cells using spinning-disk confocal microscopy and quantified the mitochondrial networks in 3D using our MitoGraph image processing method. We found that mutants lacking α -CTT contain fewer, less branched mitochondria resulting in a 20% decreased mitochondrial to cell volume ratio compared to WT cells. This suggests that α -CTTs are required to maintain mitochondrial content, most likely by regulating mitochondrial biogenesis pathways. α -CTT mutant cells also display wider, swollen mitochondrial tubules in respiring cells. This suggests that α -CTT mutant cells may have altered internal ultrastructure organization, which we are verifying by electron microscopy. These phenotypes are unique to α -CTT – mutants lacking β -CTT contain normal mitochondrial content, but have misshapen and occasionally aggregated tubules. Furthermore, cell growth rate and mitochondrial membrane potential are not affected in respiring α -CTT mutant cells, indicating that mitochondria remain functional, despite defects in content and morphology. To define the role of α -CTT in mitochondrial biogenesis and morphology, we examined the sequence features of α -CTT that are necessary for function. We find that α -CTT function can be rescued by mutants that lack negatively charged residues in α -CTT or by replacing α -CTT with the longer, and more negatively charged, β -CTT. These data suggest that α -CTT function depends on the length of the tail, but not its charge. We are currently investigating the molecular mechanisms behind tubulin-CTT regulation of mitochondrial biogenesis and morphology.

P2130**Board Number: B653****Regulation of Mitochondrial Release from Microtubules during Cell Division.**J. Chung^{1,2,3}, J.A. Steen², T.L. Schwarz^{2,3};¹Biological and Biomedical Sciences, Harvard Medical School, Boston, MA, ²The F.M. Kirby Neurobiology Center, Boston Children's Hospital, Boston, MA, ³Department of Neurobiology, Harvard Medical School, Boston, MA

During mitosis, daughter cells depend on proper inheritance of both chromosomes and organelles. Cytoskeletal rearrangement during mitosis is therefore accompanied by changes in organelle behavior and organelle interactions with the cytoskeleton. The mechanism behind ER, Golgi apparatus, and nuclear envelope regulation have been examined, but to date, the mechanism behind mitochondrial redistribution and inheritance remains unknown. During interphase, mitochondria move along microtubules via a motor-adaptor complex, consisting of Miro (also called RhoT1/2), which localizes to the mitochondrial outer membrane; the adaptor protein Milton (also called Trak1/2); and the conventional Kinesin-1 Heavy Chain and Dynein motors. During interphase, most of a cell's mitochondria are therefore closely associated with microtubules. However, as the cell enters mitosis, the mitochondria detach from microtubules and do not appear to associate with either the spindle apparatus or astral microtubules. In HeLa and COS cells, mitotic mitochondria reside primarily in a ring towards the periphery of the cells, do not overlap the spindle, and remain unattached to microtubules until late in anaphase. We have found that mitochondrial disassociation is a consequence of the detachment of the kinesin and dynein motor proteins from their adaptors Miro and Milton. Attaching motors onto mitochondria independent of Miro and Milton mislocalizes the mitochondria onto the mitotic spindle, subsequently causing mitochondrial inheritance and spindle orientation defects. Furthermore, *in vitro* kinase and phosphatase assays determined that motor release is caused by CDK1 phosphorylation of mitochondrial proteins. An attractive target may be the motor adaptor Milton. We have found that Milton is highly phosphorylated and consequently exhibits a cell-cycle dependent band shift on western blots. Milton phosphorylation is a likely trigger for the shedding of the motor proteins from the mitochondrial surface and consequent dissociation of mitochondria from mitotic microtubules. The dissociation of mitochondria from mitotic microtubules is likely to be important for proper inheritance of chromosomes and mitochondria.

P2131**Board Number: B654****Inner membrane fusion mediates spatial distribution of axonal mitochondria.**Y. Yu¹, H. Lee¹, J. Suhan¹, G. Yang¹;¹Carnegie Mellon University, Pittsburgh, PA

The morphological and motility dynamics of mitochondria are striking. It is thought that mitochondrial motility is important for mediating the spatial distribution of the organelle in neurons. However, whether and, if so, how morphological dynamics play a role in mediating axonal mitochondrial distribution is unclear. To address this question, we analyzed the relations between mitochondrial inner membrane fusion and the spatial distribution of axonal mitochondria. We used *Drosophila* larval motor neurons axons as the model system. We collected time lapse images of mitochondria in proximal and distal axon regions. For each mitochondrion, we characterized its movement and shape, which allowed us to perform correlation analysis between the two dynamic processes. We found that knockdown of mitochondrial inner membrane protein dOpa1 led to fragmentation of both stationary and moving mitochondria, with stationary mitochondria affected more substantially. We characterized mitochondrial morphological dynamics in larval motor neuron axons, confirmed that mitochondria underwent frequent fusion and fission in axons. Loss of dOpa1 blocked axonal mitochondrial fusion and reduced fission. Furthermore, we analyzed mitochondrial shape, movement and localization in different regions along the axon. We found that dOpa1 deficiency disrupted spatial distribution of axonal mitochondria. Mitochondria failed to localize to the distal axon regions, but accumulated in the proximal axon regions in dOpa1 knockdown axons. Together, our data revealed a strong connection between mitochondrial inner membrane fusion and spatial distribution.

P2132**Board Number: B655****TRPM2 channels regulate organelle zinc homeostasis and mitochondrial fragmentation.**N. Abuarab^{1,2}, J. Li¹, L. Jiang¹, A. Sivaprasadarao¹;¹Multidisciplinary Cardiovascular Research Centre, School of Biomedical Sciences, University of Leeds, Leeds, United Kingdom, ²College of Medicine, King Saud Bin Abdulaziz University for Health Science-National Guard Hospital, Jeddah, Saudi Arabia

Mitochondria play a central role in oxidative stress induced cell death. By increasing the production of reactive oxygen species, such as H₂O₂, oxidative stress causes mitochondrial fragmentation and apoptosis. Here we hypothesised that Transient Receptor Potential Melastatin 2 (TRPM2) channels play a role in mitochondrial fragmentation. The rationale behind this is the previous evidence that TRPM2 channels are activated by H₂O₂ and conduct ions (Ca²⁺ and Zn²⁺) that affect mitochondrial health and cell survival. To test our hypothesis we have used human umbilical vein endothelial cells (HUVECs). Using FluoZin-3, we demonstrate the presence of free Zn²⁺ in lysosomes, but no detectable free Zn²⁺ in

other compartments of the cell. Exposure of HUVECs to H₂O₂ (1 mM) or high glucose (33 mM) conditions led to lysosomal membrane permeability (LMP), resulting in a marked decrease in lysosomal Zn²⁺ and, interestingly, redistribution of Zn²⁺ to mitochondria. The resultant rise in mitochondrial Zn²⁺ led to extensive mitochondrial fragmentation, mitochondrial outer membrane permeabilisation and cell death. Importantly, release of lysosomal Zn²⁺ was dependent on Ca²⁺ entry through the plasma membrane TRPM2 channels. Silencing of TRPM2 channels with siRNA prevented intracellular Zn²⁺ redistribution, mitochondrial fragmentation and cell death. We found that H₂O₂ causes mitochondrial fragmentation by promoting the recruitment of dynamin-related protein 1 (Drp-1, a mitochondrial fission protein) to mitochondria. TRPM2 siRNA and the Zn²⁺ chelator, TPEN (N,N,N',N'-tetrakis (2-pyridinylmethyl)-1,2-ethanediamine), inhibited Drp-1 recruitment as well as mitochondrial fragmentation. Taken together, our data imply a novel mechanism for how oxidative stress leads to excessive mitochondrial fragmentation and cell death: The mechanism involves activation of TRPM2 channels leading to increased Ca²⁺ entry, translocation of lysosomal Zn²⁺ to mitochondria, Drp-1 recruitment to mitochondria, mitochondrial fragmentation and finally cell death. The physiological relevance of TRPM2 role was established by the demonstration that TRPM2 siRNA inhibits mitochondrial fragmentation in human endothelial cells induced by hyperglycaemic (diabetes) conditions. Since mitochondrial fragmentation is associated with several age-related chronic illnesses including neuronal (Alzheimer's, Parkinson's), cardiovascular (atherosclerosis, myocardial infarction) and metabolic/inflammatory (diabetes) disorders, our results reveal TRPM2 channel as a novel target that could be explored for therapeutic intervention of age-related illnesses.

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P2133

Board Number: B656

Cardiolipin phase transition induced by Dynamin-related protein 1 promotes mitochondrial membrane fission.

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Cardiolipin is a unique, dimeric phospholipid essential for mitochondrial dynamics in eukaryotic cells. Here, using model membranes and a variety of novel fluorescence spectroscopic and microscopic techniques, we show that the mitochondrial fission GTPase, dynamin-related protein 1 (Drp1) associates with sequestered but uncomplexed cardiolipin molecules at the mitochondrial surface and reorganizes them in a nucleotide-dependent manner upon helical self-assembly. We demonstrate further that Drp1-mediated cardiolipin reorganization results in the lipid's rapid phase transition from a lamellar phase to the non-lamellar, inverted hexagonal phase. GTP-dependent cardiolipin phase transition effected by Drp1 results in the formation of localized membrane constrictions that become predisposed for fission. We propose that Drp1 thus catalyzes mitochondrial division.

P2134**Board Number: B657****Spire1C promotes actin- and ER-mediated mitochondria constriction and fission.**U. Manor¹, S. Bartholomew², H. Higgs³, J.A. Spudich², J. Lippincott-Schwartz^{4,5};¹Cell Biology and Metabolism, NIH/NICHD, Bethesda, MD, ²Stanford University, Stanford, CA,³Department of Biochemistry, Geisel School of Medicine at Dartmouth College, Hanover, NH,⁴Physiology Course at Marine Biological Laboratory, Woods Hole, MA, ⁵Cell Biology and Metabolism Program, NICHD, NIH, Bethesda, MD

Mitochondria must regularly undergo fission in order to maintain proper cellular health; impaired mitochondria fission can lead to a plethora of diseases ranging from cancer to neurodegeneration. The molecular mechanism by which mitochondria fission is coordinated with the rest of the cell remains unclear, although a role for actin polymerization has been previously implicated. Recent work suggests that the endoplasmic reticulum (ER) induces mitochondria constriction as a necessary step for mitochondria fission; it is thought that this constriction enables Drp1, a dynamin-related GTPase protein, to oligomerize into a helical ring around mitochondria and induce membrane scission at these constriction sites. This model was corroborated by later work, which showed that the actin-polymerization activity of the ER-anchored formin protein INF2 enhances mitochondria constriction and fission. However, the specific mechanism by which INF2 and actin is recruited to and activated at mitochondria fission sites is unknown. Here we show that a previously undiscovered alternate splice isoform of the actin-nucleating, formin-binding Spire protein localizes to mitochondria. Using structured illumination microscopy and the fluorescence protease protection assay (FPP), we show that this novel isoform, Spire1C, localizes to the mitochondrial outer membrane. Using the FPP assay, we also show that the exon unique to this isoform inserts through the mitochondrial outer membrane with the N-terminal side facing the cytoplasm, and the C-terminal side facing the mitochondrial lumen, and that it binds to cardiolipin. We also found that Spire1C N-terminal KIND domain binds to the C-terminal half of INF2 in vitro, and that removing the KIND domain from Spire1C strongly inhibits mitochondria constriction and fission, whereas overexpression of Spire1C enhances constriction and fission. Disrupting Spire1C actin-nucleating ability similarly disrupts mitochondria fission. While overexpression of a Spire1C KIND domain deletion mutant results in decreased association between mitochondria and the ER, overexpression of full length Spire1C increases association between ER-anchored INF2 and mitochondria. Interestingly, overexpression of the KIND domain deletion mutant did not disrupt Drp1 localization to mitochondria, suggesting that the prior step of constriction is required for proper Drp1-mediated mitochondria fission. These results strongly suggest that Spire1C facilitates coordination of the ER and actin to enhance mitochondria fission. These results show, for the first time, that two halves of an actin-regulatory complex can reside at the interface between the membranes of two different organelles, thereby presenting a novel mechanism by which cellular organelles can communicate with and shape one another via the actin cytoskeleton.

P2135**Board Number: B658****Dynamin-related protein 1 (Drp1), inhibited by its A-insert, utilizes mitochondrial fission factor (Mff) as a GTPase-activating protein (GAP).**P. Macdonald¹, N. Stepanyants¹, L. Lehman¹, A. Baglio^{1,2}, R. Ramachandran¹;¹Physiology and Biophysics, Case Western Reserve University, Cleveland, OH, ²Case Western Reserve University, Cleveland, OH

Eight different isoforms or splice variants of the mitochondrial fission GTPase, dynamin-related protein 1 (Drp1) arise from alternative splicing of its single gene-encoded pre-mRNA transcript. Of these, the longest isoform of Drp1 bears a unique stretch of amino acid residues within its GTPase domain termed the "A-insert", whose function is yet not known. By comparing the properties of this neuronally enriched isoform with that of the ubiquitously expressed Drp1 isoform in which this sequence is absent, the role of the A-insert in Drp1 function is revealed. Utilizing a variety of biochemical and biophysical measures, we find that the A-insert auto-inhibits Drp1's helical self-assembly as well as cooperative GTPase activity over mitochondrial membranes, without impairing GTP binding, thus necessitating the requirement for an external GTPase-activating protein (GAP) to relieve this auto-inhibition. The MOM-integrated Drp1 adaptor protein, mitochondrial fission factor (Mff), fulfills such a role by selectively, and potently, stimulating the longest Drp1 isoform's GTPase activity independent of its interaction with the consensus mitochondrial target lipid, cardiolipin (CL). Thus, Mff functions selectively as a GAP for A-insert-auto-inhibited Drp1.

P2136**Board Number: B659****Mitochondrial remodeling protein Opa1-like influences *Drosophila* epithelial tissue homeostasis and stem cell maintenance.**S. Banerjee¹;¹Department of Biological Sciences, Tata Inst Fundamental Res, Mumbai, India

Mitochondrial remodeling proteins with conserved functions in regulating fusion and fission, have a role in implicating cellular/tissue homeostasis and are thus of immense interest. Mitochondrial remodeling proteins like Dynamin related protein-1 (Drp1), Mitofusin-2 (Mfn-2) and Optic atrophy1 (OPA1) have been shown to be involved in cellular homeostasis and stem cell differentiation. I'm interested in understanding the implication of changes in mitochondrial morphology has on tissue homeostasis. Here, using proteomics approach, we identified bonafide and unknown interactors of *Drosophila* Optic atrophy1-like (Opa1-like) such as *Drosophila* Mitofilin (dMitofilin), Mitochondrial assembly regulatory factor (Marf), Rhomboid-7 (Rho-7) along with novel interactors like Mortalin ortholog (Hsc70-5). Further, we showed how Opa1-like influences mitochondrial morphology through interaction with

'mitochondria-shaping' proteins like Marf (Mitofusin ortholog in *Drosophila*) and dMitofilin. Depletion of *hsc70-5* in peripodial cells, yields fragmented, dysfunctional mitochondria (with Opa1-like being proteolysed) that were susceptible to degradation leading to induced lysosome-mediated cell death. Similar phenotype was observed in *hsc70-5* knockdown precursor stem cells (AMPs-Adult Midgut Progenitors) of larval gut which were reversed by changing the mitochondrial morphology via manipulation of mitochondrial remodeling proteins. To understand the significance of death of AMPs that bring about altered tissue behavior and homeostasis, I investigated the role for mitochondrial remodeling proteins in maintaining adult midgut stem cell niche. *Drosophila* adult midgut homeostasis is maintained by Intestinal Stem Cells (ISCs) that divide to form diploid progenitors known as enteroblasts (EBs) which further differentiate into enteroendocrinal (ee) cells and enterocytes (ECs). Depletion of those proteins that regulate mitochondrial morphology and metabolism in ISC/EB cells resulted in fragmented mitochondria with increased number of ee cells and clustered EBs, a hallmark of Notch activity deficient gut. Moreover, these depletions led to increased ROS-induced hyper-proliferation of ISCs along with hyper-activation of EGFR signaling and early-onset of intestinal hyperplasia. Both hyper-proliferation and hyper-activation of EGFR requires Yorkie function. Finally, using genetic epistasis and clonal analysis, I demonstrated that Opa1-like gain of function reverses the cytological dysregulation through change in mitochondrial morphology. Thus, mitochondrial role in the maintenance of adult fly gut stem cell niche cross-talks with that of Notch and Hippo-EGFR pathways. These findings uncover a role for mitochondrial remodeling leading to cell growth/death in tissues.

P2137

Board Number: B660

Structure and dynamics of Miro, a target for PINK1/Parkin signaling of mitochondrial autophagy.

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The outer mitochondrial membrane protein Miro is a highly conserved calcium-binding GTPase that is a key player in regulating mitochondrial shape, movement, organelle interactions, and degradation. We solved and published an X-ray crystal structure of a *Drosophila* Miro fragment containing the EF hand and C-terminal GTPase domains (termed EF-cGTPase). This is the first protein structure containing interacting EF hand and GTPase domains. The structure reveals putative PINK1 kinase phosphorylation sites and Parkin-ubiquitinated sites in and near the interface between Miro's EF hand region and the C-terminal GTPase domain. We have new data suggesting that this interface is highly dynamic. While full-length constructs of both *Drosophila* Miro and human Miro-1 are monomeric, an isolated fragment of the human Miro-1 C-terminal GTPase (cGTPase) forms a homo-dimer. We observed this homo-dimer formation using analytical SEC-MALS, SAXS, and in new X-ray crystal structures that our lab has solved. Interestingly, the interface between the dimerized cGTPase proteins is in the same region as the EF hand-GTPase interface in our previous EF-cGTPase structure. It is possible that the EF hand/C-terminal

GTPase interaction and/or dimerization of the C-terminal GTPase may protect Miro from ubiquitination until it becomes phosphorylated by PINK1 kinase. These data are beginning to provide a structural basis for Miro-dependent PINK1/Parkin signaling of mitochondrial autophagy.

P2138

Board Number: B661

The mitochondrial peptidase subunit alpha defines PINK1 alternative mitochondrial import route.

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PINK1, a Parkinson's disease associated gene encoding a mitochondrial kinase, in concert with *Parkin*, another PD gene that encodes an E3 ubiquitin ligase, mediate the autophagy of damaged mitochondria. Loss of the mitochondrial membrane potential, defects in mitochondrial import and the activation of the mitochondrial unfolded protein response are stimuli that lead to PINK1 stabilization on the outer mitochondrial membrane, which in turn recruits Parkin from the cytosol to the mitochondria in order to initiate mitophagy. In healthy cells, PINK1 is processed by multiple proteases in the mitochondria followed by proteasomal degradation in the cytosol by the N-end rule pathway. However, the specific manner and route by which PINK1 is imported and degraded remains to be clarified. We have recently shown that PINK1 also accumulates at the mitochondrial outer membrane following the partial depletion of the catalytic, β subunit of the mitochondrial processing peptidase (MPP). Indeed, MPP β knockdown recapitulates the main features of PINK1/Parkin-dependent mitophagy induced by chemical uncouplers. Here, we report that PINK1 contains an N-terminal MTS that is processed directly by MPP both *in vitro* and *in vivo*. Moreover, we show that the regulatory subunit MPP α , is required for PINK1 insertion and accumulation at the outer mitochondrial membrane both upon MPP β knockdown and mitochondrial depolarization but is not necessary for canonical PINK1 import to the inner mitochondrial membrane. Taken together, our works positions MPP α as a key regulator of PINK1 import, required to divert PINK1 to the outer mitochondrial membrane and to initiate parkin-dependent mitophagy in response to insults that compromise mitochondrial health.

P2139**Board Number: B662****Parkin regulates the activity of MiTF/TFE transcription factors independent of mTORC1 during mitophagy.**C. Nezhich^{1,2}, A.I. Fogel¹, C. Wang¹, R.J. Youle¹;¹NINDS, NIH, Bethesda, MD, ²MRC Mitochondrial Biology Unit, University of Cambridge, Cambridge, United Kingdom

Loss-of-function mutations in the mitochondrial kinase PINK1 and the cytosolic E3 ligase Parkin cause the majority of early-onset familial Parkinson's disease cases. Together, PINK1 and Parkin regulate a quality-control pathway, termed mitophagy, which selectively eliminates damaged mitochondria by autophagosome-dependent lysosomal degradation. Coordinated expression of autophagy and lysosomal genes is regulated by the transcription factor TFEB; however, TFEB has yet to be linked to mitophagy. Because mitophagy requires robust lysosomal function, we sought to understand the molecular link between mitophagy and lysosomal biogenesis. Here, we show that during mitophagy TFEB translocates from the cytosol to the nucleus, where it is active, in a PINK1 and Parkin-dependent manner. Unlike during starvation-induced autophagy, this activation of TFEB was independent of mammalian target of rapamycin complex I (mTORC1) inhibition. To examine whether TFEB was required for mitophagy we generated TFEB null cells using CRISPR-mediated genomic editing. Unexpectedly, these cells exhibited no defect in mitophagy and, contrary to previous studies that used RNAi to decrease TFEB levels, genomic elimination of endogenous TFEB also had no apparent effect on lysosomal morphology. Exploring the potential for compensation for TFEB loss by other members of the MiTF/TFE transcription factor family (MITF, TFEC, and TFE3), we observed Parkin-dependent nuclear translocation of MITF and TFE3 during mitophagy, similar to TFEB. We generated single knockout cell lines for the remaining MiTF/TFE family members and found that each exhibited a partial mitophagy defect that was further enhanced when two or more MiTF/TFE family members were knocked out in the same cell line. This indicates that members of the MiTF/TFE family coordinately participate in mitophagy. Our identification of PINK1 and Parkin as novel regulators of MiTF/TFE family member activation, via a unique pathway that works independently of mTORC1, illuminates a much more dynamic regulation of their activity than previously appreciated and provides new insight into the molecular mechanisms that link mitophagy and lysosomal biogenesis.

P2140**Board Number: B663****PINK1 is a ubiquitin kinase.**L.A. Kane¹, M. Lazarou¹, A.I. Fogel¹, Y. Li¹, K. Yamano¹, S.A. Sarraf¹, S. Banerjee¹, R.J. Youle¹;¹NINDS, NIH, Bethesda, MD

PINK1 is a gene mutated in certain autosomal recessive early onset forms of Parkinson's disease (PD). During the decade since its identification, it has been shown to be a mitochondrial kinase whose

abundance is controlled by mitochondrial membrane potential. Low mitochondrial membrane potential, signaling an unhealthy organelle, causes the accumulation of PINK1 on the outer mitochondrial membrane. This results in the recruitment of Parkin, a cytosolic E3 ubiquitin ligase that is also mutated in early onset PD. Parkin then induces the recruitment of both the proteasome and machinery responsible for autophagy, thus facilitating turnover of damaged mitochondria. The substrates of PINK1 have been a source of debate and many substrates have been suggested in the literature, including Parkin itself. Using an unbiased proteomic approach, we have now identified that PINK1 directly phosphorylates ubiquitin (Kane, *et al. J. Cell Biol.* 205, 143-153 (2014)). This phosphorylation occurs specifically on Ser65 of ubiquitin and mirrors PINK1's known phosphorylation of the ubiquitin-like domain of Parkin. Phospho-Ser65 ubiquitin binds to Parkin and activates its E3 ligase activity *in vitro*. Overexpression of a non-phosphorylatable mutant ubiquitin blocks Parkin translocation to damaged mitochondria *in vivo*. This is the first identification of a ubiquitin kinase and the first reported function for phospho-ubiquitin. Phosphorylation of ubiquitin in this clinically-relevant mitochondrial pathway opens the potential for a broader cellular signaling role for ubiquitin post-translational modification.

P2141

Board Number: B664

Deoxynucleoside salvage and compartmentalization in regulating mitochondrial deoxynucleoside triphosphates levels.

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Mitochondrial DNA depletion diseases have been identified, in which there is a failure in maintaining mitochondrial biogenesis due to deficiencies and/or mutations of deoxynucleotide salvage pathway enzymes. However, little is known concerning the role of the salvage pathways in regulating deoxynucleosides (dNs) metabolism and deoxynucleoside triphosphate (dNTP) pool sizes, particularly in tissues that are not replicating nuclear DNA and are much more dependent on the salvage pathway. Mitochondrial dysfunction has been associated with a variety of anti-cancer and anti-viral nucleoside analogs that may adversely affect the salvage pathway in non-replicating tissues. The role of protein transporters in moving precursors, intermediates and products of dNTP synthesis is still largely unknown. The purpose of this study is to determine the role of exogenous deoxynucleoside concentration on the size of dNTP pools and the compartmentation of these pools in isolated intact rat heart mitochondria. To accomplish this goal, freshly isolated rat heart mitochondria were incubated at 30°C for 60 minutes with increasing concentrations of tritium-labeled or unlabeled deoxynucleosides. After incubation, mitochondria were separated from the medium by centrifugation. In some cases, mitochondria were further fractionated into membrane and soluble fractions through repeated freezing and thawing. The synthesis of the labeled deoxynucleotides was measured by separation using UPLC and an in-line liquid scintillation counter. The synthesis of dNTPs from unlabeled deoxynucleosides was measured in a modified template driven dNTP assay. Results obtained from current investigation

indicated that the mitochondrial level of TTP and dGTP present were highly sensitive to the exogenous thymidine and deoxyguanosine concentration respectively while the level of dCTP and dATP were insensitive to the exogenous deoxycytidine and deoxyadenosine concentration respectively. Thymidine was the preferred substrate over deoxycytidine for thymidine kinase (TK2) and deoxyguanosine was the preferred substrate over deoxyadenosine for deoxyguanosine kinase (dGK). Further, thymidine was much preferred over TMP as substrate for the synthesis of TTP. The TTP synthesized appeared to equilibrate readily with the medium, while surprisingly, dGTP, being 90% of the total dNTP pools, was highly concentrated inside the mitochondria and remained with membrane fraction on mitochondrial breaking. In conclusion, regulation of the salvage pathway in supporting mitochondrial dNTP pools appears to be compartmentalized and different to each deoxynucleosides.

P2142

Board Number: B665

Calorie restriction: Effects of oxidative and nitrosative stress at the mitochondrial level.

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Calorie restriction (CR) is an experimental manipulation without malnutrition that is known to extend the lifespan of a number of organisms including yeast, worms, flies, rodents and non-human primates. In addition, CR has been shown to reduce the incidence of aging-related disorders (for example, diabetes, cancer and cardiovascular disorders) in mammals. The mechanisms through which this occurs have been unclear. The aim of this project is to determine the effects of a prenatal and perinatal calorie restriction in rats on oxidative and nitrosative stress in liver mitochondria. Female Wistar rats of 200-300 g of body weight were used and were caged individually and maintained in a 12:12 (light:dark) cycle at a 22 +/- 2 °C. The control groups were fed ad libitum and restricted animals received daily 50% of control animal's food intake (50% calorie restriction). After two weeks of treatment the female rats were caged with males for one week and mating was confirmed by the presence of spermatozoa in a vaginal smear. Pregnant rats were submitted to calorie restriction during the course of gestation and during lactation period, pups were calorie restricted too. At the age of one, three and five months the animals restricted were measured and weighed before the sacrifice by decapitation. The liver was immediately processed to isolate mitochondria. Results obtained showed variation depending the age-development of the rats. An increase on mitochondrial nitric oxide and lipoperoxidation in the perinatal and postnatal stages were observed, as well as peroxynitrite synthesis, respect control. Presence of antioxidants as glutathione and glutathione reductase activity were measured. Both antioxidants measurements were decreased both in pre and postnatal stages. These results suggest that CR has negative control over oxidative and nitrosative stress in the perinatal stage. Acknowledgements: The authors appreciate the partial economic support of the grant of CIC-UMSNH (2.16).

P2143**Board Number: B666****Mitochondrial dynamics in astrocytic processes during secondary pathology in an *in vitro* model of transient ischemic stroke.**J.C. O'Donnell^{1,2}, J.G. Jackson¹, M.B. Robinson^{1,2};¹Pediatrics, Children's Hospital of Philadelphia, Philadelphia, PA, ²Pharmacology, University of Pennsylvania, Philadelphia, PA

Astrocytes are the most abundant cell type in brain. They are responsible for clearing extracellular glutamate, the predominant excitatory neurotransmitter, from the synapse to maintain crisp signaling and prevent excitotoxicity. In forebrain, the astrocytic glutamate transporter, GLT-1, is responsible for the vast majority of glutamate uptake. Mitochondria are invested throughout fine astrocytic processes where they colocalize with GLT-1. We recently discovered physical and functional interactions between GLT-1, multiple glycolytic enzymes, and mitochondria. We also found that mitochondria in astrocytic processes are retained near glutamate transporters and synapses. Our data suggest that this distribution is regulated by neuronal glutamate release, astrocytic glutamate uptake, and reversal of the Na⁺/Ca²⁺ exchanger. Mitochondria can support glutamate uptake by providing ATP and buffering ions, and a portion of transported glutamate is oxidized in mitochondria to generate energy in these compartments.

Mitochondrial dysfunction and excitotoxicity from failure of astrocytic glutamate uptake are at the core of delayed cell death that occurs after a transient ischemic insult. Aside from inducing hypothermia, this secondary pathology is currently untreatable. The goal of this project is to quantify changes in astrocytic mitochondrial distribution during the secondary pathology period in an *in vitro* model of stroke. To do so we have employed transient oxygen glucose deprivation (OGD) in organotypic hippocampal slice cultures biolistically transfected to fluorescently label astrocytic mitochondria. In ongoing experiments we have observed a loss of mitochondria in astrocytic processes in response to OGD that precedes the peak of delayed neuronal cell death common to this model and to stroke *in vivo*. In preliminary experiments, cyclosporin A, which increases mitochondrial capacity for calcium buffering, reduced cell death and attenuated the loss of mitochondria in astrocytic processes after OGD. Preliminary observations also provide evidence for increased mitophagy (a mechanism for degradation of dysfunctional mitochondria) in astrocytic processes after OGD. Further investigation of the mechanisms underlying the loss of astrocytic mitochondria after transient OGD could provide new therapeutic targets for the currently untreatable secondary pathology following transient ischemic stroke.

P2144**Board Number: B667*****In vivo* tissue-wide synchronization of mitochondrial metabolic oscillations.**N. Porat-Shliom¹, Y. Chen², A. Shitara¹, M. Tora¹, O. Harding¹, A. Masedunskas¹, R. Weigert¹;¹NIDCR, NIH, Bethesda, MD, ²NCI, NIH, Bethesda, MD

Little is known about the spatio-temporal coordination of mitochondrial metabolism in multicellular organisms *in situ*, and how oxidative phosphorylation adapts to rapid changes in energy demand during processes such as membrane trafficking. To address this issue we investigated how mitochondrial metabolism is modulated during GPCR-stimulated exocytosis in the acinar cells of salivary glands (SGs) in live rats. Specifically, we developed a method to follow the dynamics of the mitochondrial metabolic activity in live rodents that is based on the use of intravital two-photon microscopy to determine NADH levels (the main substrate of the electron transport chain, ETC) and mitochondrial potential. Unexpectedly, we discovered that mitochondrial metabolism undergoes rapid and spontaneous oscillations in SGs under basal conditions (period: 10-15 sec), in contrast with what reported in *ex vivo* or *in vitro* model systems where transient metabolic oscillations are observed only after agonist stimulation. These oscillations are regulated by reactive oxygen species but are insensitive to modulations in the levels of intracellular Ca²⁺. Most notably, we found that mitochondria *in vivo* behaves as a network of functionally coupled oscillators, which maintain a high level of coordination throughout the tissue. Indeed, mitochondria in different acini oscillates with the same period and phase, and their coordination is disrupted by blocking the activity of gap junctions. Finally, we observed that β -adrenergic stimulation, which stimulates regulated exocytosis, transiently halts NADH oscillations and increases mitochondrial potential, thus generating the ATP necessary to complete secretion. The mechanism regulating this process will be discussed in here. In conclusion, the ability to measure and characterize metabolic oscillations through NADH emission *in vivo*, has revealed novel aspects of cellular metabolism that have not been appreciated in reductionist model systems, and will open new avenues to study mitochondrial metabolism and tissue energetics at a cellular, subcellular, and tissue scale both under physiological and pathological conditions.

P2145

Board Number: B668

Characterization of the zebrafish family of Trak proteins.

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Mitochondria serve as the site of oxidative phosphorylation and can move to areas that possess high energetic needs within a cell. This movement is necessary to maintain basic metabolic functions, and disruption of this motility often results in cell death. In fruit flies and mammals, Miro, an outer mitochondrial membrane protein, and Trak, a kinesin adaptor protein, comprise one protein complex that regulates mitochondrial trafficking along microtubules. Our research aims to determine the role of the zebrafish Trak family of proteins. Through sequence homology, we have identified three zebrafish Trak proteins: zTrak1, zTrak2, and zTrak1-like. When expressed in COS7 cells, both human Trak1 and Trak2 are mitochondrial. However, in COS7 cells, zTrak1 does not localize to the mitochondria, yet zTrak2 does. A chimeric protein containing the N-terminus of zTrak1 and the C-terminus of human Trak1 relocated to the mitochondria, indicating that the C-terminus of hTrak1 is sufficient to direct

mitochondrial localization, and the C-terminus of zTrak1 is likely responsible for its cytosolic localization. Given the low amino acid conservation among these paralogs, these zebrafish proteins might have different cellular functions, and we are currently exploring the role of these proteins in vivo using antisense morpholino oligonucleotides and RNA in situ hybridization in developing zebrafish embryos. Taken altogether, this research will lay the foundation for studies in the role of mitochondrial motility during vertebrate development.

Kinases and Phosphatases

P2146

Board Number: B670

Reconstitution of Apoptotic Cell Engulfment to Decipher an Ancient Signaling System.

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Apoptosis is crucial during development and for maintaining healthy adult tissues. However, the appearance of apoptotic cells is normally rare: dying cells are efficiently recognized, internalized, and digested by phagocytes (a process termed corpse clearance). Since inefficient corpse clearance leads to inflammation and further tissue damage, a mechanistic understanding of how engulfment works and how the process can be activated may spur development of rational therapies for inflammatory diseases.

Elegant genetic work has identified the components of an ancient signaling system conserved across flies, worms and mammals that drives engulfment. However how these factors work together to drive the process remains unclear. We have reconstituted apoptotic cell engulfment by a *Drosophila* pathway driven by the receptor Draper and kinase Shark. Expression of Draper in cultured cells converts inept phagocytes into efficient engulfing cells. Recognition and engulfment of apoptotic cells or physiological mimics is rapid and specific.

A clue to the mechanism of Draper and Shark may lie in the signaling pathway that triggers T cells. In the latter case, the T cell receptor complex (TCR) works with a kinase ZAP-70 at the membrane. Both apoptotic cell engulfment and T cell triggering occur at sites of membrane-membrane contact two cells and require actin. Furthermore the molecules that drive each process bear remarkable similarity. We have shown that Shark, like ZAP-70, is recruited to the plasma membrane during signaling. Similar to TCR and ZAP-70, Draper and Shark form clusters at the plasma membrane that undergo retrograde flow, indicating that functional properties of the signaling systems are conserved between these divergent pathways.

We have established a live cell imaging platform to interrogate the contribution of specific molecules towards efficient engulfment; in particular we hope to understand how the Draper pathway interfaces

with downstream actin machinery. As a similar system has been proposed to function in mammalian cells, we look forward to extending our findings from *Drosophila* to other organisms.

P2147

Board Number: B671

Amyloid β -Induced Activation of mTOR at the Plasma Membrane Leads to Neuronal Cell Cycle Re-Entry: a Seminal Step in Alzheimer's Disease Pathogenesis.

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Mechanistic Target of Rapamycin (mTOR) plays a major role in cell metabolism by sensing availability of resources, such as growth factors and amino acids, and integrating the resulting signals to control cell growth and proliferation. These functions involve finely tuned regulation of mTOR activity at specific membrane locations, and mTOR dysregulation has been implicated in cancer and Alzheimer's disease (AD).

AD is characterized by the accumulation in brain of two types of insoluble deposits, extracellular plaques composed of amyloid- β (A β) peptides and intraneuronal neurofibrillary tangles comprising the microtubule-associated protein, tau, and by massive synaptic dysfunction and neuron death. Ironically, a large fraction of neuron death in AD occurs after the normally post-mitotic neurons ectopically re-enter the cell cycle. We recently reported that neuronal cell cycle re-entry (CCR) results from a process by which soluble A β oligomers (A β Os) stimulate PKA, fyn and CAMKII to catalyze site-specific tau phosphorylation (Seward, et al. 2013. J Cell Sci 126:1278). We now describe further details of the CCR pathway, including a novel and essential role for mTOR.

Ki-67 and Cyclin D1 expression, or BrdU uptake were used to identify cultured WT mouse neurons that respectively had exited G0, were in G1, or had entered or completed S-phase after exposure to A β Os. CCR was prevented by knockdown of NCAM, Gas, RalA, Rac1 or eIF4E, or reducing the activity of mTORC1 or mTORC2 by respective knockdown of raptor or rictor. Pharmacological inhibition of mTOR or Rac1, or exposure of neurons to a cell permeable peptide that blocks Rac1 binding to mTOR also prevented CCR. By using a genetically encoded mTORC1 activity reporter (Zhou, et al. Cell Metab *in revision*) we detected A β O-induced mTOR activation at neuronal cell membranes, but not in lysosomes. Reducing Rac1-dependent translocation of mTOR to plasma membrane (PM) domains and forcing mTORC1 association to lysosomes also prevented CCR. In vivo studies showed that CCR marker expression in cortical neurons of Tg2576 AD model mice was strongly suppressed in animals with

genetically-reduced mTor activity. Finally, we found that this Rac1-modulated mTOR signaling controls *in vitro* and *in vivo* tau phosphorylation at S262, which is also necessary for neuronal CCR.

A β O_s thus initiate two pathways that together lead to mTOR dysregulation and are obligatory for ectopic neuronal CCR. One pathway mistargets Rac1-mTOR complexes to the PM, and the other leads from G α s and NCAM to activation of fyn, PKA and CaMKII, which then respectively phosphorylate tau at Y18, S409 and S416. The CCR requirement for tau phosphorylation at S262 establishes that regulation of tau and mTor by each other integrates the two pathways.

P2148

Board Number: B672

BAG-1 differentially regulates intermediate filament-based Hsp70 chaperoning of aPKC in intestinal cells under pro-inflammatory signaling.

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Atypical protein kinase C (aPKC) plays an essential role in the establishment of epithelial polarity and tight junction assembly. aPKC acquires inactive conformation after normal activity and can be rescued from ubiquitinylation and degradation by Hsp-70- and intermediate filament-dependent mechanism. We have shown previously that aPKC was strongly downregulated by TNF-alpha-mediated signaling in intestinal epithelial cells and also *in vivo* during intestinal inflammation. Furthermore we have demonstrated that decrease of aPKC levels under pro-inflammatory conditions was mediated through inhibition of Hsp70 chaperoning activity, resulting in failure of the aPKC rescue machinery. The goal of this work was to identify the molecule (or molecules) responsible for Hsp70 inhibition under TNF-alpha stimulation. First, we conducted a transcriptome PCR screen detecting chaperones and co-chaperones and compared mRNAs from control and TNF-alpha treated Caco-2 cells (human colon carcinoma). Subsequent validation experiments allowed us to focus on BAG-1, a multi-functional protein that assists Hsp70 in nucleotide exchange but also blocks its activity at higher concentrations. We found that BAG-1 isoform BAG-1M was upregulated up to 3 fold in human Caco-2 cells following stimulation with TNF-alpha. In addition, BAG-1M levels increased up to 6 fold in mouse enterocytes following treatment with dextran sodium sulfate (DSS) to induce colitis. BAG-1M, but no other isoform, was found to co-purify with intermediate filaments. To test the effect of BAG-1M on Hsp70 chaperoning activity we employed a well-established chemically denatured luciferase refolding assay. Addition of BAG-1M to the assay reaction within the range of concentrations found in epithelial cells resulted in significant inhibition of Hsp70 chaperoning activity in the intermediate filament fraction. To determine if the findings observed *in vitro* also apply to the living cells, we have performed both overexpression and knockdown of Bag-1M in Caco-2 cells using lentiviral transduction. Overexpression of BAG-1M decreased levels of phosphorylated aPKCs, similar to TNF-alpha stimulation. In contrast, knockdown of BAG-1 abolished the TNF-alpha-induced decrease of phosphorylated aPKC. Overall, we conclude that BAG-1M mediates inhibition of Hsp70 chaperoning activity during epithelial inflammatory response.

P2149**Board Number: B673****Optogenetic modulation of Raf/ERK and PI3K/AKT pathways in PC-12 cells - understanding the temporal dimensions of antiapoptotic pathways against oxidative stress.**Q. Ong¹, K. Zhang^{1,2}, S. Guo¹, L. Duan¹, E.A. Collier¹, B. Cui¹;¹Chemistry, Stanford University, Stanford, CA, ²Molecular and Cellular Biology, University of Illinois at Urbana-Champaign, Urbana, IL

Intracellular optogenetics offers chemical biologists an unprecedented new set of tools in manipulating and probing signaling pathways with spatiotemporal precision. We have utilised the light protein-interaction pair, CRY2PHR and CIBN, to elicit Raf and AKT recruitment to the plasma membrane via blue light controllably, with reversible dissociation under dark conditions. This optogenetic toolkit is being applied to study how Raf/ERK and PI3K/AKT pathways act as protective mechanisms to the cells under oxidative stress (hydrogen peroxide and singlet oxygen generation), with greater understanding towards their temporal dimensions.

P2150**Board Number: B674****The MLK3-mediated positive feedback loop is a crucial factor in cellular responses to reactive oxygen species.**C. Hwang¹, H. Lee¹, K. Cho¹;¹Department of Bio and Brain Engineering, Korea Advanced Institute of Science and Technology, Daejeon, Korea

Reactive oxygen species (ROS) play a central role in the regulation of numerous cellular processes. It is known that opposite cellular response such as proliferation or cell cycle arrest/apoptosis can be made depending on ROS concentration. However, it remains unclear how such different cellular response can be determined through a complex signaling network. In this study, we found that extracellular signal-regulated kinases (ERKs) and c-Jun N-terminal kinases (JNKs) coordinate ROS-dependent cellular response determination. ERKs mediate proliferation at low ROS concentration, whereas JNKs mediate cell cycle arrest/apoptosis at high ROS concentration. From mathematical simulations combined with biochemical experiments, we found that mixed lineage kinase 3 (MLK3)-mediated positive feedback loop balances the signal flow for proliferation and cell cycle arrest/apoptosis, which results in contrasting cellular response determination. Cells treated with an MLK3 inhibitor or MLK3 shRNA showed increased viability at high ROS concentrations through higher ERKs activation and lower JNKs activation than control cells. Taken together, our systems biology analysis and biochemical studies provide new insight into the mechanism underlying how ROS-dependent cellular response is determined.

P2151**Board Number: B675****Anti-inflammatory Effects of Heparin on Vascular Endothelial Cells are Dependent on Dual-Specificity Mitogen-Activated Protein Kinase Phosphatase-1.**S.N. Farwell¹, J.B. Slee^{1,2}, L.J. Lowe-Krentz³;¹Lehigh University, Bethlehem, PA, ²Pediatrics, Children's Hospital of Philadelphia, Philadelphia, PA,³Biological Sciences, Lehigh University, Bethlehem, PA

Heparin is widely-used as an anti-coagulant as part of treatment for increasingly prevalent cardiovascular and pulmonary diseases. Anti-proliferative effects of heparin on vascular smooth muscle cells (VSMCs) have been well documented and involve both the down-regulation of the mitogen-activated protein kinase (MAPK) intermediate Erk and the up-regulation of dual-specificity MAPK Phosphatase-1 (MKP-1). Heparin has been also shown to exhibit potent anti-inflammatory properties on vascular endothelial cells (ECs) in vitro, but the molecular mechanism(s) by which heparin accomplishes this remain unclear. We examined heparin's effects on primary macrovessel ECs. We tested the hypothesis that heparin's effects are also at least partially dependent on increased MKP-1 expression in ECs. We show that heparin decreases tumor necrosis factor (TNF) α -induced activation of stress-activated protein kinases (SAPKs) c-Jun N-terminal kinase (JNK) and p38 through western blotting and immunofluorescence assays. Using knockdown strategies, we show these effects are partly dependent on MKP-1. Heparin-induced decreases in TNF α -induced actin stress fiber production also require an MKP-1 induction, as shown through quantitative fluorescence microscopy. An advanced understanding of heparin's anti-inflammatory effects is crucial to identify novel heparin responsive targets and to develop and improve therapeutic strategies. Exploiting heparin as a drug with anti-inflammatory actions is an important direction to pursue in both cardiovascular research and clinical settings. Support from NIH award HL54269 to LLK.

P2152**Board Number: B676****Metabolic control of Ca²⁺/Calmodulin-dependent protein kinase II (CaMKII)-mediated Caspase 2 suppression by the B55 β /protein phosphatase 2A (PP2A).**B. Huang¹, C. Yang¹, J. Wojton¹, N. Huang¹, C. Chen¹, E.J. Soderblom¹, S. Kornbluth¹;¹Duke University Medical Center, Durham, NC

High levels of metabolic activity confer resistance to apoptosis. Caspase 2, an apoptotic initiator, can be suppressed by high levels of nutrient flux through the pentose phosphate pathway (PPP). This metabolic control is exerted via inhibitory phosphorylation of the caspase 2 pro-domain by activated Ca²⁺/Calmodulin-dependent protein kinase II (CaMKII)(1). We show here that metabolic activation of CaMKII depends, in part, on dephosphorylation of CaMKII at novel sites (T393/S395) and that this is

mediated by metabolic activation of protein phosphatase 2A (PP2A) in complex with the B55 β targeting subunit. This represents a novel locus of CaMKII control and also provides a mechanism contributing to metabolic control of apoptosis. These findings may have implications for metabolic control of the many CaMKII-controlled and PP2A-regulated physiological processes, as both enzymes appear to be responsive to alterations in glucose metabolized via the PPP.

1. Nutt, L. K., Margolis, S. S., Jensen, M., Herman, C. E., Dunphy, W. G., Rathmell, J. C., and Kornbluth, S. (2005) Metabolic regulation of oocyte cell death through the CaMKII-mediated phosphorylation of caspase-2. *Cell* 123, 89-103

P2153

Board Number: B677

Functional interaction network of the conserved NDR kinase Orb6.

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Cell polarization is the common feature of a variety of different cell types and is fundamental to many cellular processes, including cell differentiation, cell motility and cell fate determination. Defects in cell morphogenesis are linked to diseases such as cancer, developmental defects and neuronal disorders. The conserved NDR kinase Orb6 controls cell morphogenesis in different organisms, ranging from yeast to neuronal cells. In mammals, NDR kinase is also thought to function as a tumor suppressor. Our lab has previously shown that the conserved NDR kinase homologue in fission yeast, Orb6 kinase, plays a key role in maintaining polarized cell growth at the cell tips by spatially restricting the localization and activity of Cdc42 GTPase.

To identify novel functional targets of NDR kinase Orb6, we used an ordered fission yeast gene deletion library to perform a genome-wide screen for gene functions that display synthetic lethality or suppression interactions with an inhibitor-sensitive Orb6 kinase mutant. This innovative screen further refined the screen results using computational, mass spectrometry, and 2-hybrid analysis approaches. Refined gene candidates fall into four major functional groups, including morphogenesis, nutritional response, transcriptional and translational control. Of particular interest are novel genetic, functional and physical interactions of Orb6 kinase with conserved protein complexes involved in the control of transcription and heterochromatin gene silencing.

P2154**Board Number: B678****An endoplasmic reticulum lipid phosphatase regulates plasma membrane lipid metabolism.**E.J. Dickson¹, J.B. Jensen¹, O. Vivas¹, B. Hille¹;¹Physiology and Biophysics, University of Washington, Seattle, WA

Phosphoinositides are a family of low-abundance phospholipids located on the cytoplasmic leaflet of cellular membranes that maintain cell structure, cell motility, membrane trafficking, ion channel functions and also play key roles in signal transduction. Phosphatidylinositol (4,5)-bisphosphate (PI(4,5)P₂) is the signature phosphoinositide of the plasma membrane. How is PM PI(4,5)P₂ sourced? We have reported that plasma membrane PI(4,5)P₂ levels are supported by at least two, continuously supplying, precursor pools of PI(4)P, one in the plasma membrane itself, and the other in the Golgi. How is plasma membrane PI(4,5)P₂ regulated? Many lipid phosphatases and kinases are needed for the maintenance of each cellular phosphoinositide pool; we focused our attention on an integral membrane lipid 4-phosphatase enzyme localized to the endoplasmic reticulum (ER), Sac1. It has the ability to reduce the precursor source of PM PI(4,5)P₂, namely PI(4)P. Using a variety of optical (confocal, TIRF, super-resolution microscopies), protein dimerization, and electrical (ion channel recordings) techniques, we have discovered that Sac1 aggregates in puncta, intimately apposed to the plasma membrane in endoplasmic reticulum–plasma membrane (ER-PM) contact sites in tsA-201 cells. Based on FRET measurements between Sac1 and a plasma membrane marker, Sac1 is within 10 nm of the plasma membrane. What causes Sac1 to aggregate in ER-PM contact sites? Multiple lines of evidence suggest a role for the cortical ER protein, extended synptotagmin-2 (E-Syt2). Quantitative super-resolution imaging analysis reveals strong correlation between Sac1 and E-Syt2 distributions. Overexpressing or knocking down E-Syt2 increased or decreased the abundance of Sac1 in close proximity to the PM, respectively, and lead to alterations in plasma membrane PI(4)P and PI(4,5)P₂ levels. Activation of G-protein coupled receptors to deplete plasma membrane PI(4,5)P₂ causes a dynamic redistribution of Sac1 puncta away from the plasma membrane. The reaggregation of Sac1 back into ER-PM contacts is dependent on the rate of PI(4,5)P₂ resynthesis. Similar results were observed in superior cervical ganglia neurons. Thus, the intimate organization of ER Sac1 next to the plasma membrane gives it the unique ability to act as a cellular ‘thermostat’ controlling plasma membrane PI(4)P and PI(4,5)P₂ levels and with that many plasma membrane proteins.

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P2155**Board Number: B679****Distinct patterns of phosphatase activation and subcellular localization impact the kinetics of interferon-gamma signaling in CNS neurons.**C. Brown¹, A. Stahl¹, C. Stotesbury¹, K. Sweeney¹, G. Rall², W. Rose¹;¹Department of Biology, Arcadia University, Glenside, PA, ²Immune Cell Development and Host Defense Program, Fox Chase Cancer Center, Philadelphia, PA

The immune cytokine interferon-gamma (IFN-g) plays an essential role in the elimination of numerous central nervous system (CNS) viral infections. The cellular response to IFN-g, mediated by JAK/STAT signal transduction, involves the upregulation of genes that result in the establishment of the antiviral state, and is controlled by multiple negative feedback mechanisms. However, whether these control mechanisms, previously characterized in non-neuronal cells, operate similarly in CNS neurons has not been well-characterized. Our lab previously demonstrated that the control of the neuronal IFN-g pathway is distinct from that in non-neuronal cells, as the duration of STAT1 phosphorylation and IFN-g responsive gene expression in IFN-g treated neurons was markedly extended as compared to mouse embryonic fibroblasts (MEF). We also showed that compared to MEF, STAT1 dephosphorylation was delayed in IFN-g treated neurons, providing a mechanism for the extended kinetics of neuronal STAT1 phosphorylation. The current study investigated the expression and subcellular localization of two STAT1 phosphatases, TC45 and SHP-2, to address the hypothesis that mislocalization of these phosphatases could result in the observed delay in neuronal STAT1 dephosphorylation. Although no differences in overall expression levels of TC45 were observed between neurons and MEF, neurons demonstrated an approximately 3-fold lower level of SHP-2 activation as compared to MEF, regardless of IFN-g exposure. To examine the subcellular localization of these phosphatases, neurons and MEF were exposed to IFN-g, and nuclear and cytoplasmic fractions were purified at 1, 24, and 48h post-IFN-g treatment. Our results showed that while SHP-2 was found strictly in the cytoplasmic fraction of both cell types, TC45 was found only in the cytoplasmic fraction of the neurons, whereas it was equally distributed between nuclear and cytoplasmic fractions in MEF. These results indicate that neurons (but not MEF) lack TC45 in the nucleus, which provides a likely explanation for the observation that neuronal STAT1 is not dephosphorylated as rapidly as it is in MEF. Understanding the control of the IFN-g response in CNS neurons will ultimately aid in the characterization of antiviral immune mechanisms of the CNS.

P2156**Board Number: B680****A-Kinase Anchoring Proteins, Phosphorylated-Akt/PKB and Phosphodiesterase-4A: Players in Schwann Cell Proliferation.**A.L. Asirvatham¹, K. Spears¹, R. Stahl², D. Carey²;¹Biology, Misericordia Univ, Dallas, PA, ²Geisinger Health Center, Weis Center for Research, Danville, PA

Proliferation of Schwann cells in the peripheral nervous system is dependent upon the heregulin/neuregulin family of growth factors secreted by neurons. The stimulation of Schwann cell division by heregulin occurs in a synergistic fashion only if pathways involving cAMP are also triggered. The molecular mechanisms involved in the synergistic modulation of Schwann cell proliferation by heregulin and cAMP pathways are unknown. Previous studies have shown that A-Kinase anchoring proteins (AKAPs) of the cAMP/PKA signaling pathway play a key role in Schwann cell division. Treatment of neonatal Schwann cell cultures with SiRNA oligos synthesized against AKAP150 and AKAP95 exhibited a significant reduction in cell growth, expression of AKAPs and the cell survival signal, Akt/Protein kinase B. Based on these studies it was hypothesized that the expression of AKAP95 and AKAP150 along with the expression of phospho-Akt/PKB and the cAMP-terminating enzyme, PDE4A would increase when Schwann cells were treated with heregulin, forskolin or heregulin + forskolin. Immunoblot analysis revealed that heregulin treatment of Schwann cells increased the protein expression of AKAP95 and phosphorylated-Akt while forskolin treatment caused an upregulation in the levels of AKAP 150, PDE4A and Akt. To corroborate the results from immunoblot analysis, immunofluorescence experiments in Schwann cells were conducted, which revealed that in comparison to control, cells treated with heregulin and heregulin + forskolin exhibit distinct colocalization patterns for AKAPs and Akt while cells treated with forskolin show colocalization for AKAPs and phospho-Akt. These preliminary observations suggest that a relationship between expression of AKAP proteins and phosphorylation of Akt along with PDE4A may be necessary to mediate mitogen-stimulated Schwann cell growth.

P2157**Board Number: B681****Novel substrates of Rho kinase in the heart.**Y. Yura¹, T. Bando², M. Amano¹, K. Kaibuchi³;¹Cell Pharmacology, Nagoya University School of Medicine, Nagoya City, Japan, ²Cell Pharmacology, Nagoya University School of Medicine, Nagoya, Japan, ³Nagoya University School of Medicine, Nagoya, Japan

Rho kinase/ROCK/ROK is highly expressed in the cardiac muscle and is activated in failing heart. However, the substrate and function of Rho kinase in the heart have not been reported so far. We previously developed a novel proteomic approach for screening of protein kinase substrates by combining mass spectrometry and affinity column chromatography of catalytic domain of protein kinase. Using this method, we searched for novel substrates of Rho kinase in the rat heart tissue, and

more than 200 candidate substrates were obtained. We then confirmed that some of these candidate substrates including cardiomyopathy-related gene products were really phosphorylated by Rho kinase in vitro. Among them, we focused on ANKRD1/CARP. ANKRD1 has been reported to play a critical role in the maintenance of sarcomere integrity and transcriptional regulation of cardiac genes dependent on its subcellular localization. We further searched for the binding partners whose interactions are affected by phosphorylation state of ANKRD1. Among the known partners, transcriptional factor YB-1 preferentially bound to non phosphorylated ANKRD1 rather than phosphorylated one. In addition, we also found that 14-3-3 proteins specifically associated with phosphorylated ANKRD1. These results suggest that Rho kinase modifies the ANKRD1 function by regulating its transcriptional activity and subcellular localization through the phosphorylation.

P2158

Board Number: B682

Exploring signaling through TAK1 during SAN development.

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Following a myocardial infarction, cardiomyocytes in the damaged heart tissue are replaced by collagen and fibroblasts. This structural change leads to a decrease in cardiac function and also arrhythmia. One developing treatment option for this damaged tissue is the cellular transplantation of cardiac cells derived from pluripotent cells such as embryonic stem (ES) cells. However, this therapy will require a deeper understanding of the mechanisms that determine cardiac cell fates. Previous work in the Foley lab has shown that early cardiac formation is dependent upon intracellular signaling between the endoderm and mesoderm, mediated by antagonists of the canonical Wnt/ β -Catenin signaling pathway and activators of the Nodal signaling pathway. TGF β -activated kinase (TAK1), which is located directly upstream of the Wnt antagonist NLK, is thought to be critical for cardiac differentiation toward the sinoatrial node (SAN) fate. We hypothesize that SMAD-independent TGF β signaling through TAK1/p38/JNK or TAK/NLK pathways influences formation of the heart inducing endoderm and indirectly to direct SAN differentiation within the myocardium. This study focused on the role of TAK1 in mouse ES cells. Mouse ES cells were transduced with the TAK1 gene and the TAK1 ES cells were then differentiated as embryoid bodies (EBs). Western blots compared protein content of different signaling markers of TAK1 cells to unmodified ES cells. Continuous TAK1 mRNA overexpression and the effects on patterning were assessed by qRT-PCR. Markers for the endoderm and the sinoatrial node were significantly upregulated while cardiac contractile proteins were downregulated. In addition we found TAK1 cells alter the expression of Jnk and NLK in ES cells. Therefore, we have determined that TAK1 overexpression could be directing pacemaker differentiation by acting through one or both of these downstream targets.

P2159**Board Number: B683****Activin-Beta/TGF-Beta signaling in skeletal muscle controls insulin signaling and metabolism to influence final body size.**L. Moss-Taylor¹, M. O'Connor¹;¹Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN

Body size is tightly regulated during development to maximize adult fitness. In *Drosophila*, final body size is mainly determined by the growth rate and the duration of growth during juvenile stages; once maturation occurs, body size is set. The rate of growth is determined by insulin-like peptide (dilp) signaling and metabolism, while the duration of growth is regulated by prothoraciotropic hormone (PTTH) and ecdysone. Manipulation of these pathways alters final body size by either accelerating or delaying the onset of metamorphosis. Skeletal muscle is known to coordinate tissue and body growth during development, influencing final size. Levels of insulin signaling in skeletal muscle proportionately affect body and organ size, but what influences muscle-specific insulin signaling and how the muscle coordinates tissue growth are unknown.

We are investigating the newly identified role that the TGF-Beta ligand Activin-Beta plays in regulating *Drosophila* body size and timing of metamorphosis. We have found that mutations in dActivin-Beta (dAct-Beta) cause accelerated pupariation and reduced final body and organ size. To determine how Act-Beta affects size and timing, we first looked at which cells express Act-Beta and found expression in the Insulin Producing Cells (IPCs), neuroendocrine cells and motor neurons. Overexpression of Activin-Beta in either neuroendocrine cells or motor neurons increases body size. Muscle-specific knockdown of the TGF-Beta signaling transducer/transcription factor *dSmad2* reduces body size, indicating muscle is a target tissue of the Activin-Beta signal. Additionally, levels of phospho-dSmad2 are reduced in skeletal muscle samples of Activin-Beta mutants and increased in animals overexpressing Activin-Beta from motor neurons. Levels of phospho-S6K are correlated with phospho-dSmad2 levels, suggesting TGF-Beta signaling in muscle regulates muscle-specific insulin signaling. Because insulin signaling controls metabolism, we used GC/MS analysis to identify and quantify levels of metabolites in whole-larval samples of Activin-Beta mutants. We found intermediates of the energy-producing steps of glycolysis and lactic acid are reduced, indicated reduced flux through glycolysis. Overall, this indicates neuronally-derived Activin-Beta signals to the skeletal muscle to regulate levels of insulin signaling and subsequent glycolysis. We will identify downstream targets of dSmad2 using skeletal-muscle RNAseq and test potential target genes using tissue-specific knock-downs to determine how TGF-Beta signaling influences insulin signaling and how the muscle coordinates body growth and final organ size.

P2160**Board Number: B684****Understanding the mechanisms of cellular growth control with: (1) the Rose Rosette Virus in plants and (2) Angiotensin II controlled Hippo signaling in mammals.**J. Adler¹, C. Wells²;¹Brescia University, Owensboro, KY, ²Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN

Rose Rosette Virus: Various cultivars of Rose are currently under attack by the Rose Rosette Virus. This emerging virus is threatening the existence of many Rose cultivars, by inducing rapid proliferation leading to the death of the plant. The Adler laboratory is currently interested in growth control of these plants and any differences between the unaffected and those infected with the virus. We hypothesize that the virus is working through a mechanism that promotes aggressive uncontrolled growth, and we are exploring the role that this virus may be playing to hijack growth control pathways, similar to those seen in mammalian cancer cells.

Angiotensin II: Mammalian cellular growth is controlled by microenvironmental signals in serum under both normal physiological settings and during cancer progression. Importantly, the effects of several of these microenvironmental signals are mediated by the activities of the tumor suppressor protein kinases of the Hippo pathway. Canonically, Hippo protein kinases inhibit cellular growth through the phosphorylation and inactivation of the oncogenic transcriptional co-activator Yes-Associated Protein (YAP). In the Wells laboratory, we defined an alternative mechanism whereby Hippo protein kinases induce growth arrest via the phosphorylation of the long isoform of Angiotensin II (Amot130). Specifically, serum starvation is found to activate the Hippo protein kinase, Large Tumor Suppressor (LATS), which phosphorylates the adapter protein Amot130 at serine-175. Importantly, wild-type Amot130 potently inhibits epithelial cell growth, unlike the Amot130 serine-175 to alanine mutant, which cannot be phosphorylated at this residue. The growth-arrested phenotype of Amot130 is likely a result of its mechanistic response to LATS signaling. Specifically, LATS activity promotes the association of Amot130 with the ubiquitin ligase Atrophia-1 Interacting Protein 4 (AIP4). As a consequence, the Amot130-AIP4 complex amplifies LATS tumor suppressive signaling by stabilizing LATS protein steady state levels via preventing AIP4-targeted degradation of LATS. Additionally, AIP4 binding to Amot130 leads to the ubiquitination and stabilization of Amot130. In turn, the Amot130-AIP4 complex signals the ubiquitination and degradation of YAP. This inhibition of YAP activity by Amot130 requires both AIP4 and the ability of Amot130 to be phosphorylated by LATS. Together, these findings significantly modify the current view that the phosphorylation of YAP by Hippo protein kinases is sufficient for YAP inhibition and cellular growth arrest. Based upon these results, the inhibition of cellular growth in the absence of serum more accurately involves the stabilization of Amot130 and LATS, which together inhibit YAP activity and epithelial cell growth.

P2161**Board Number: B685****Tight control of PP2A holoenzyme biogenesis and catalytic subunit recycling.**Y. Xing¹;¹Oncology, University of Wisconsin-Madison, Madison, WI

The function of metalloenzyme protein phosphatase 2A (PP2A) relies on proper formation of diverse heterotrimeric PP2A holoenzymes, comprising common scaffold and catalytic (PP2Ac) subunits and a variable regulatory subunit. Holoenzyme assembly is highly regulated by carboxymethylation of PP2Ac. Our previous study demonstrated that the enzymatic reaction of PP2A methylation is highly responsive to PP2A activation via a direct binding of PP2A methyltransferase to the dynamic PP2A active site (Mol. Cell. 2011, 41(3):331-42). Our recent studies showed that PP2A holoenzyme biogenesis requires PP2Ac stable latency and activation. We showed that the entire protein fold of PP2Ac is highly dynamic and tends to partially unfold in the absence of catalytic metal ions, which renders α 4-binding that involves the inner structure of PP2Ac near the active site. α 4-binding leads to an allosteric relay of conformational changes that perturbs the scaffold subunit binding site at the opposite surface. This unique mode of α 4-binding underlies important mechanisms for stable latency of PP2Ac as well as dissociation of PP2A holoenzymes and recycling of PP2Ac in response to cell signaling. Due to the flexible nature of the apo-PP2Ac, the protein fold of apo-PP2Ac needs to be stabilized by PP2A phosphatase activator (PTPA) via broad contacts with the structural elements surrounding the PP2A active site, which stabilizes the PP2Ac active site in a conformation that could bind catalytic metal ions. PTPA-binding also defines a combined ATP-binding pocket that orients ATP phosphoryl groups to bind directly to the PP2Ac active site. This allows ATP to modulate the metal-binding preferences of the PP2Ac active site and drastically enhances binding of Mg²⁺ by 10,000-fold, which is crucial for acquisition of pSer/Thr-specific phosphatase activity. Consistent with the activation chaperone function of PTPA and activation-dependent PP2A methylation, PTPA stimulates both PP2A phosphatase activity and methylation. Collectively, our studies showed that PP2A stable latency, activation, methylation, and holoenzyme assembly are elegantly coordinated by conformational switches of PP2Ac, underlying novel mechanisms for tight control of PP2A function and regulation in context of cellular signaling.

P2162**Board Number: B686****Real-time Dynamics of the Purinosome Governed by AMPK-associated Signaling Network.**D.L. Schmitt¹, S. An²;¹Chemistry and Biochemistry, Univ Maryland- Baltimore County, Baltimore, MD, ²Chemistry and Biochemistry, Univ Maryland Baltimore County, Baltimore, MD

The objective of this study is to determine if signaling networks associated with AMP-activated protein kinase (AMPK) implicate purinosome dynamics in cancer cells. Human *de novo* purine biosynthesis is a ten-step process catalyzed by six enzymes, using phosphoribosyl pyrophosphate to produce inosine monophosphate. These enzymes have previously been found to compartmentalize together in the cytoplasm upon purine depletion; forming a transient metabolic complex termed the “purinosome.” Recently, there has been growing interest in elucidating signaling networks associated with purinosome dynamics to understand the biological significance of purinosome assembly in cells. Although a metabolic intermediate of purine biosynthesis, 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR), is a well-characterized allosteric activator of AMPK, human *de novo* purine biosynthesis has not yet been explored as part of AMPK-mediated signaling circuits. In this study, we have investigated the effect of AMPK activation on purinosome dynamics by monitoring green fluorescent protein-tagged formylglycinamide ribonucleotide synthase (FGAMS-GFP) as a purinosome marker under fluorescence live-cell microscopy. Dynamic clustering of purinosomes is observed in ~70% of HeLa cells by the addition of AICAR. Subsequently, we reveal that the biguanides, metformin and phenformin, also induce clustering of FGAMS-GFP in ~79% and ~75% of HeLa cells, respectively. These data support our hypothesis that AMPK controls purinosome assembly as a mean to maintain cellular energy homeostasis. We will continue to delineate the coupled network between the purinosome and AMPK in various cancer cell lines by fluorescence live-cell microscopy in combination with established biochemical assays. As *de novo* purine biosynthesis has been a validated target of cancer therapeutics, we envision that a greater understanding of purinosome dynamics, especially in response to cellular energy needs, possesses high potential to be translated into better chemotherapeutic intervention.

P2163**Board Number: B687****Regulation of IP6K2 activity by TRAF2.**H. Rahman^{1,2}, G.J. Miller²;

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Overexpression of inositol hexakisphosphate kinase-2 (IP6K2) sensitizes multiple cancer cell lines to the apoptotic effect of IFN- α 2, IFN- β , TNF α , γ -irradiation and other apoptotic inducing agents, while its knockdown confers resistance to these stressors. IP6K2 binds to tumor necrosis factor receptor associated factor-2 (TRAF2), which serves as an adapter protein for the TNF-receptor superfamily and interleukin-1 receptor and is a major mediator for the regulation of cell survival and cell death for these receptors. Two short amino acid motifs (SXXE) in IP6K2 have previously been shown to be responsible for TRAF2 binding, which inhibits phosphorylation of TAK1 and AKT, thereby inhibiting NF- κ B activation and promoting apoptosis. Our objectives are (1) to characterize the interaction of each IP6K2 motif with TRAF2, (2) determine the impact of this interaction on IP6K2 catalytic activity and (3) determine the structural basis of this interaction.

P2164**Board Number: B688****Analysis of secreted protein kinase substrates.**A. Sreelatha¹, K. Orth¹, J. Dixon², V. Tagliabracci²;

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Kinases mediate protein phosphorylation of intracellular and secreted proteins to regulate various cellular processes. Until recently, the kinases that phosphorylate secreted proteins were unidentified. One member of the atypical secreted protein kinase family, Fam20C phosphorylates the S-x-E/pS motif of over 100 secreted proteins. Genetic diseases such as Raine syndrome that result from Fam20C mutations highlight the importance of Fam20C phosphorylation of secreted protein. Further analysis of Fam20C substrates reveal that it also regulates wound healing and lipid homeostasis. Using bioinformatics, biochemistry and genetics we study the substrates of Fam20C kinases and the role of phosphorylation on substrate function.

P2165**Board Number: B689****Mechanisms and functional consequences of T cell microclusters**X. Su¹, J. Ditlev², E. Hui³, J. Rumpf³, S. Banjade², J. Taunton³, M.K. Rosen^{4,5}, R. Vale³;¹Department of Cell and Molecular Pharmacology, University of California, San Francisco, San Francisco, CA, ²University of Texas Southwestern Medical Center, Dallas, TX, ³University of California, San Francisco, San Francisco, CA, ⁴Department of Biophysics, University of Texas Southwestern Medical Center, Dallas, TX, ⁵Howard Hughes Medical Institute, Dallas, TX

The assembly of higher-order protein structures has been suggested to promote signaling whereas a clear cause-and-effect relationship has been difficult to establish, largely due to limited knowledge about how these structures are formed. To investigate the mechanisms and functional properties of membrane-bound signaling clusters, we developed an in vitro reconstitution system in which signaling clusters are assembled from individual purified components on synthetic supported lipid bilayers. We found multivalent protein-protein interactions can drive the cluster formation of LAT, a signaling hub protein that recruits multiple downstream effectors for TCR signaling. LAT clusters enrich Sos1(RasGEF), PLCg1(Phospholipase), and Nck1(connecting to actin polymerization). Clustering renders resistance to dephosphorylation of LAT by protein tyrosine phosphatase, which suggests a mechanism of how clustering might maintain TCR signaling. In addition, we found two adaptor proteins Grb2 and Gads are redundant and competitive for clustering LAT. Finally, through reconstituting a signaling pathway from TCR triggering to LAT clustering, and to actin polymerization, we showed LAT cluster nucleates actin filaments and promotes membrane-proximal actin network formation. Together, we propose TCR triggering induces a phase transition of LAT complex that promotes downstream signaling.

P2166**Board Number: B690****Identification of Novel Oncogenic Drivers in Cancer Cell Lines Through a Systems Pharmacology Approach.**N. Sakur¹, N. Salmen¹, M.Y. Fink¹;¹LIU Post, Brookville, NY

Oncogenes drive cancer cell growth and identification of novel candidate oncogenes is a necessary step for developing new drugs. Integration of data from genomic, proteomic, and literature findings, in cancer cell lines, can generate models of altered signaling networks in different cancer types and subtypes. Several large-scale systems biology studies have screened cancer cell lines for drug sensitivity. In this study, systems-level network models of cancer cell lines have been generated and, based on these models, drug sensitivity predictions have been made. These predictions were then compared to known sensitivities from published studies. The results identified unexpected interactions between cell

line networks and drugs. The ZR-75-30 breast cancer cell line displayed sensitivity to sorafenib, a multi-kinase inhibitor, that targets several receptor tyrosine kinases and RAF. This reported sensitivity has been confirmed and the specific target and pathway affected in ZR-75-30 cells is currently being explored.

P2167

Board Number: B691

The role of Sp1 in p21^{CIP1} regulation in B-Raf^{V600E} cancer.

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We have previously reported that mortalin, an Hsp70 family chaperone, can suppress p21^{CIP1} expression in certain B-Raf^{V600E} cancer cells. We observed that mortalin depletion in B-Raf^{V600E} cancer cells results in p21^{CIP1}-mediated cell cycle arrest in a MEK-ERK dependent manner. We thus proposed that mortalin upregulation is a potential mechanism for certain B-Raf^{V600E} cancer cells to bypass growth arrest caused by aberrant MEK-ERK activation. Interestingly, mortalin depletion could induce p21^{CIP1} expression independently of p53 in certain B-Raf^{V600E} cancer cells. In this study, we investigated the mechanism by which mortalin regulates p21^{CIP1} expression. Using luciferase reporter truncation analysis, we have identified a proximal p21^{CIP1} promoter region responsive to mortalin depletion in p53 mutated cancer cells. Interestingly, when Sp1 transcription factor-responsive elements in this region were mutagenized, the p21^{CIP1} promoter reporter was no longer responsive to mortalin depletion. Consistent with this, our ChIP analysis revealed that mortalin knockdown could induce Sp1 binding to p21^{CIP1} promoter in a MEK-ERK dependent manner. Furthermore, RNA interference of Sp1 substantially attenuated p21^{CIP1} expression induced by mortalin depletion in these cells. In summary, our data demonstrate that, in B-Raf^{V600E} cancer cells, Sp1 can regulate p21^{CIP1} transcription even in the absence of wild type p53. This study suggests that Sp1 may have an important role in p21^{CIP1} regulation in certain cancer types.

P2168

Board Number: B692

Pseudophosphatase MK-STYX regulates RhoA signaling, and enhances branching in PC12 cells.

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The pseudophosphatase MK-STYX (mitogen activated kinase phosphoserine/threonine/tyrosine binding protein) is a member of the dual specificity MAPK phosphatases (MKP) that is catalytically inactive due to mutations in its active site (HCX5R to FSX5R). Previously, we reported that MK-STYX induces neurite-like outgrowths in PC12 cells and enhances the effects of NGF stimulation by dramatically increasing the

number of neurites. Furthermore, MK-STYX induced neurite formation in the presence of a MEK inhibitor, suggesting that MK-STYX exerts its effects independently of the extracellular signaling related kinase ERK pathway. RhoA activity assays showed that MK-STYX decreases RhoA activation, whereas knockdown of MK-STYX increased RhoA activation- suggesting that MK-STYX is a regulator of RhoA signaling. Here, we further investigate the role of MK-STYX as a regulator of RhoA signaling by examining MK-STYX's effects on cofilin, a RhoA downstream effector. Cofilin is an actin binding protein that depolymerizes actin, resulting in cytoskeletal reorganization. MK-STYX did not alter cofilin expression but did alter its phosphorylation. Phosphorylated cofilin (p-cofilin) stabilizes actin filaments, causing persistence of neurites and continued growth. MK-STYX caused an increase in phosphorylated cofilin over time after NGF stimulation, whereas, levels of p-cofilin in control cells stayed consistent over time. Furthermore, p-cofilin decreased when MK-STYX was knocked down with shRNA. Throughout the course of these experiments, we noticed that cells overexpressing MK-STYX developed more neurites, which appeared to branch. To address whether MK-STYX induced a distinct branching pattern in PC12 cells, we scored cells to categorize the neurites branching pattern. Initially, cells were scored for the presence of neurites greater than 20 μm . Cells with at least one extension meeting this criterion were scored for primary (extension from cell body) and secondary neurites (extensions from primary neurite). Cells not stimulated by NGF but expressing MK-STYX showed a slight shift in overall distribution of primary and secondary neurites compared to the control. NGF stimulated cells overexpressing MK-STYX showed a greater shift in distribution of both primary and secondary neurites compared to the control. These data demonstrate that MK-STYX causes branching, which is regulated by the Rho GTPases such as RhoA, in PC12 cells. Taken together these data provide more substantial evidence for MK-STYX as a regulator of RhoA signaling and neuronal differentiation.

P2169

Board Number: B693

Characterization of a novel yeast gene, *XRR1*, which confers resistance to the antifungal rapamycin and whose protein may be localized to stress granules.

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Cell cycle arrest in response to the macrolide antifungal agent rapamycin (sirolimus) in the yeast *S. cerevisiae* is mediated through the binding of FKBP12 protein (FPR1p) with rapamycin, forming a complex with the TOR1 and TOR2 proteins. Deletion mutations in the *FPR1* gene and specific point mutations in *TOR1* and *TOR2* genes, which prevent interaction with the FKBP12-rapamycin complex, result in a resistance to rapamycin phenotype. We have characterized a novel gene, *XRR1* (exhibits Rapamycin Resistance), whose null mutant exhibits temperature sensitive resistance to rapamycin. The *XRR1* gene product was shown via a systematic yeast two-hybrid analysis (Uetz *et al*, 2000) to physically interact with FKBP12 (FPR1p). The *XRR1* gene exhibits sequence homology to the A1pp (Macro) domain

specific for binding ADP-ribose. This domain is found in the C-terminus of the mammalian macroH2A histone variant.

Characterization of the *XRR1* gene suggests a role in the rapamycin resistance pathway. We have observed that the *XRR1* null mutation results in an eight-fold increase in growth in the presence of 100 ng/ml rapamycin at 37°C after six hours of growth compared to five-fold increase at 30°C. Wildtype isogenic strains exhibit only a 2.8 fold increase of growth under the same growth conditions.

The *XRR1* gene product may be localized to stress granules under conditions of nutritional stress. Microscopic imaging of Xrr1p-GFP indicates three subcellular distribution patterns under normal growth conditions: we have observed uniform cytoplasmic staining and staining in which the vacuolar staining is excluded in the majority of the cells observed, and punctate cytoplasmic staining in a small percentage of cells. However, under conditions of glucose starvation, we observed an increase in the punctate staining pattern and a concomitant reduction in cytoplasmic staining. This phenomenon does not appear to occur under conditions of physical stress, as we do not see an increase in punctate staining after a of 42°C heat shock.

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P2170

Board Number: B694

Calcineurin Interacting Protein works as a Modulator of tax-6 Signaling Pathway.

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Calcineurin is a serine/threonine phosphatase regulated by Ca²⁺-binding calmodulin. Calcineurin consists of two subunits, catalytic subunit A (TAX-6) and regulatory subunit B (CNB-1). Calcineurin substrate candidates of *C.elegans* have been screened by yeast two-hybrid assays. We found that CNP-2 interacted with the catalytic domain of TAX-6. The CNP-2 is expressed in head neurons, posterior intestine, spermathecal valve, spermatheca-uterus valve and cell junction/ membrane in spermatheca. In males, the CNP-2 is expressed in spicule retractor muscles, rays and at the end of fan, which are mating behavior-mediated organs. Also, CNP-2 is required in the ovulation, arrangement of eggs, ray structure and mating behaviors. CNP-2 seems to be critical in the mating efficiency and sperm activation. We are currently investigating the roles of *cnp-2* in *tax-6* pathway, regarding IP3 signaling.

P2171**Board Number: B695****Regulation of body size in C.elegans via counteracting between phosphatase and kinase.**T. Choi¹, S. Lee¹, J. Ahn²;¹BK21Plus BDR Team, Hanyang Univ, Seoul, Korea, ²Hanyang Univ, Seoul, Korea

Calcineurin, Ca²⁺/calmodulin-dependent serine/threonine phosphatase, has been reported to function in body size regulation in *C. elegans*. We confirmed neuronal function of calcineurin is important cue in the regulation of body size. RNAi screening to identify kinases which counteract with calcineurin activity revealed 33 kinases that rescued the phenotype of small body size in *tax-6(lf)* background. Among the positive candidates, we found *mbk-2* which encodes one of two *C. elegans* members of the Dual-specificity Yak1-Related Kinase (DYRK) family proteins partially rescued the body size of *tax-6(lf)*. One of the candidates target gene, *oma-1* RNAi also showed partial rescue of *tax-6(lf)*. We are doing experiments to elucidate the underlying working mechanisms.

P2172**Board Number: B696****High Glucose-Induces Oxidative Stress in HEK-293 Cells.**E. Bradshaw¹, G. Beggs¹, M. Cartafalsa¹;¹Biology, Florida Southern College, Lakeland, FL

Approximately 366 million people worldwide are affected by diabetes. A long-term complication of diabetes mellitus is kidney disease, also known as diabetic nephropathy. To date, the underlying causes of diabetes-induced kidney failure are not well understood but recent evidence points to mitochondrial dysfunction. In models of diabetic nephropathy, there is increased mitochondrial stress, which is associated with kidney cell death. In this study, HEK-293 cells were treated with 25mM glucose and ATP production and oxidative stress was assessed with an ATP assay and TBARS assay, respectively. HEK-293 cells treated with 25mM glucose for 24 hours increased ATP production as well as lipid peroxidation compared to control. These data indicate high glucose conditions influence the mitochondria in this cell type. To determine the molecular mechanism for high sugar environment changes in ATP production and oxidative stress, we examined the mTOR pathway. Recent clinical data implicates the Akt/mTOR pathway in diabetic patients with kidney disease. Preliminary western blot evidence shows that dextrose increases phosphorylation of p70S6 kinase in HEK-293 cells. This effect is inhibited by rapamycin, an inhibitor of the mTOR pathway. Thus, these data suggest mTOR may play a role in the increased ATP production and oxidative stress induced by a high sugar environment.

P2173**Board Number: B697****Regulation of DAG Lipase Activity – Implications for ‘On-Demand’ Endocannabinoid Signalling.**R.L. Markwick¹, E. Williams¹, M. Maresca², P. Singh¹, F. Howell¹, G. Williams¹, P. Doherty¹;¹Wolfson CARD, King's College London, London, England, ²Astra Zeneca, Molndal, Sweden

Diacylglycerol lipases (DAGL α and β) are key enzymes in the biosynthesis of 2-AG, the major endocannabinoid (eCB) in the brain. 2-AG acts on CB1 and/or CB2 receptors and DAGL-dependent eCB signalling regulates a large number of responses including axonal growth during development, as well as neurogenesis and retrograde synaptic plasticity in the adult (Oudin *et al.*, 2011). The enzymes also play a major role in driving pathogenic inflammatory responses via a DAGL/MAGL pathway that generates AA as a precursor to prostaglandin synthesis. DAGL antagonists are being developed as novel therapeutics based on their ability to regulate eCB-mediated signalling and/or inflammatory responses. The DAGLs appears to display ‘on-demand’ synthesis, generating increasing amounts of 2-AG in response to cellular messengers. Based on homology modelling and analysis of phosphoproteomic databases we have postulated that a phosphorylation dependent displacement of an intramolecular regulatory loop “activates” DAGL α/β activity by allowing access of the natural substrate DAG to the active site of the enzyme (Reisenberg *et al.*, 2012). We will report a cellular assay to measure DAGL α function. This assay measures DAGL-dependent activation of the CB1 receptor, reporting on physiologically relevant DAGL α activity in a cell. Evidence for phosphorylation-dependent activation of DAGL α , required for eCB signalling, and use of CRISPR/Cas9 System to systematically ‘switch off’ endogenous DAGL α , will be presented.

Oudin, M.J., Hobbs, C. & Doherty, P. (2011) DAGL-dependent endocannabinoid signalling: roles in axonal pathfinding, synaptic plasticity and adult neurogenesis. *Eur J Neurosci*, 34, 1634-1646. Reisenberg, M., Singh, P.K., Williams, G. & Doherty, P. (2012) The diacylglycerol lipases: structure, regulation and roles in and beyond endocannabinoid signalling. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 367, 3264-3275.

P2174**Board Number: B800****Analysis of the Regulatory Mechanism by which Shear Stress and Stretch Force Induce Nitric Oxide Production in Endothelial Cells.**J. Wang^{1,2}, K. Kobayashi-Kato¹, M. Hagiwara¹, N. Ishida¹, K. Matsushita^{1,2};¹Oral Disease Research, National Center for Geriatrics and Gerontology, Nagoya, Japan, ²National Center for Geriatrics and Gerontology, Obu, Aichi, Japan

Blood vessels are subjected to constant mechanical forces from blood pressure and flow, which cause internal stresses (endothelial shear stress and circumferential wall stress, respectively). The mechanical

forces not only cause morphological changes of the endothelium and blood vessel wall but also trigger biochemical and biological events. Physiologic shear stress and stretch force exert vasoprotective effects via nitric oxide (NO) and provide a homeostatic oxidative balance. Perturbation of these mechanical forces can disturb biochemical homeostasis and lead to vascular remodelling and possible dysfunction. It is known that these distinct biological endpoints are caused by some common biochemical pathways. However, little is known about the difference of biological pathways evoked by shear stress and by stretch force. In this study, we focused on NO production induced by shear stress and by stretch force and compared the regulatory mechanism of these stimuli with the NO production in endothelial cells. We examined NO production induced by short (30 sec -10 min) exposure of endothelial cells (HUVECs) to laminar shear stress and sustained stretch force. A high level of NO was produced and peaked at 1 min and 4 min after exposure to shear stress in HUVECs. On the other hand, a low level of NO was produced at 30 sec after exposure to stretch force. Chelation of intracellular Ca²⁺ with BAPTA-AM completely abolished the phosphorylation of Akt and eNOS and the production of NO induced by shear stress, whereas BAPTA-AM suppressed only half of the phosphorylation of Akt and eNOS and NO production induced by stretch force and, conversely, complete depletion of Ca²⁺ in the culture strongly enhanced NO production by HUVECs. These results suggest that the regulatory mechanism by which endothelial cells produce NO depends on the type of stress and that the role of Ca²⁺ signal in NO production may differ with stimulation from shear stress and stimulation from stretch force. The results may be useful for understanding the responses of blood vessels to internal vessel stresses induced by blood pressure and flow.

Rho-Family GTPases

P2175

Board Number: B801

A Novel Role for Wiskott - Aldrich syndrome Protein (WASP) in the Regulation of Macrophage Nanotubes.

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Macrophages are known to interact with tumor cells, pathogens, lymphocytes endothelial cells, and many other cell types via small molecule intermediaries. However, traditional soluble means of communication do not account for all macrophage interactions suggesting other means of communication. Recently discovered tunneling nanotubes (TNTs) are membranous channels that connect different cells together. These connections are thin and may be up to several cell diameters long. These structures occur in many immune cells in vitro and in vivo allowing for transfer of signals, vesicles, and organelles. Even pathogens can use these TNTs to transfer from cell to cell. Due to their length and the ability to form networks of TNTs, cell signals can be propagated over vast distances quickly and without interaction with extracellular fluid. Because these structures have no known

markers and are sensitive to light exposure, shearing force, and chemical fixation, little is known about the formation of these structures in macrophages. In this study, TNTs are characterized in macrophages by in vitro live cell imaging which minimizes TNT breakage. Live visualization of the membrane was achieved by either staining with the fluorescent membrane label, FM1-43FX, or by expression of a fluorescent protein targeted to the plasma membrane using a CAAX motif. Interestingly, macrophages polarized to either the pro-inflammatory or pro-tumorigenic states, using LPS or IL-4, show increased number of TNTs. Formation of TNTs was also investigated when actin-polymerization, PI3 kinase or Src kinase pathways were blocked. Moreover, it was determined that Wiskott-Aldrich syndrome protein (WASP) regulates formation and stabilization of TNTs. Depletion of WASP by shRNA in a macrophage cell line greatly reduces formation of these structures. Furthermore, while phosphorylation of WASP was minimally required for the formation of these structures, it was important for TNT stability. These results reveal a novel role of WASP in macrophage TNT formation. Interestingly, they also suggest that some of the immune deficiencies observed in patients with Wiskott-Aldrich syndrome may be due to the decreased number of TNTs. More research will need to be done to find potential ways to manipulate TNTs therapeutically.

P2176

Board Number: B802

Minding the GAPs: A novel RhoGAP family of RPEL proteins as target for regulation by G-actin.

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Small GTPases of the Rho family control the dynamics of the cytoskeleton through multiple effector proteins that regulate the assembly, activity and turnover of actin-based structures, thereby controlling cell adhesion, morphology and motility in response to extracellular signals. Activation of these GTPases lead to the accumulation of filamentous actin (F-actin) through both filament stabilisation and de novo polymerisation, with concomitant depletion of cellular levels of monomeric actin (G-actin). RPEL proteins are a novel family of G-actin-binding proteins, which are able to sense fluctuations in cellular G-actin concentration, and respond to maintain cytoskeleton homeostasis and remodelling. Two families of RPEL proteins have been described: the MRTF family, which couples gene transcription to F-actin availability, and the Phactr family, which coordinates actomyosin crosslinking with F-actin level.

We identified two novel RhoGAP subfamilies of RPEL proteins, among which Arhgap12 has been the focus of our study. Our data show that Arhgap12 is a GTPase activating protein towards the small GTPase Rac1. It regulates the remodelling of the actin cytoskeleton, cell motility and invasion. Moreover, direct binding of G-actin regulates its subcellular localisation and inhibits its activity by competing with Rac1 binding. Structural studies and point mutation analyses indicate that actin interacts both with the RPEL motif and with a conserved sequence embedded in the GAP domain. We

propose that Arhgap12 orchestrates a homeostatic feedback loop to coordinate G-actin level to the activation of Rac signalling.

P2177

Board Number: B803

Development of a proof-of-concept small molecule inhibitor of MgcRacGAP identifies new potential functions of MgcRacGAP in cell division and signaling.

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The MKLP1/MgcRacGAP/Ect2 protein complex is well-established as a core regulator for initiation and progression of cytokinesis. Interestingly, it also appears to be involved in oncogenesis through a cytokinesis-independent mechanism where overexpression drives invasiveness, epithelial-to-mesenchymal transition and chromosomal segregation aberrations. Furthermore, MgcRacGAP is reported to be essential for nuclear translocation of STAT transcription factors, including STAT3, which is a well-known promoter of oncogenesis and cancer drug resistance. Small molecule modulators of MgcRacGAP would be very valuable research tools for the detailed study of the many apparent roles of MgcRacGAP. Therefore, we developed a biochemical high throughput screen for identification of inhibitors of MgcRacGAP and screened 370,000 compounds. From this campaign, we identified the first publicly described small molecule RhoGAP inhibitor. This compound (MINC1) showed selective biochemical inhibition of MgcRacGAP at low micromolar concentrations. Cellular treatment with MINC1 resulted in inhibition of proliferation and increased frequencies of multinucleation, in agreement with it being a cytokinetic inhibitor. Surprisingly, we also observed collapsed mitotic spindles that resembled a failure at prophase/pro-metaphase similar to a PLK1-inhibited phenotype. Since MgcRacGAP is a substrate of PLK1, this indicates that MgcRacGAP may also play a role in PLK1-regulated mitotic processes. In agreement with MgcRacGAP regulating cellular polarity and invasion, treatment with MINC1 or siRNA-mediated knockdown of MgcRacGAP caused a strong inhibition of MDA-MB-231 breast cancer cell line matrigel invasion. Finally, based on previous published reports proposing that the MgcRacGAP-Rac1 complex plays a vital role in STAT family transcription factor nuclear translocation, we examined the capability of MINC1 to disrupt STAT signaling. Surprisingly, we instead found that both MINC1 treatment and siRNA-mediated knockdown of MgcRacGAP caused an activation of STAT3 and STAT5B signaling in several cell lines, suggesting that the proposed role of MgcRacGAP in STAT family transcription factor function may not be generally applicable. In conclusion, we have identified MINC1, a small molecule inhibitor of MgcRacGAP. This compound represents the first described selective small molecule inhibitor of a small G-protein GTPase activating protein. We utilized this inhibitor to study the function of MgcRacGAP in cell division and in other biological contexts with both expected and unexpected results. Ongoing work is focused on optimizing the compound to allow further detailed studies of the role of MgcRacGAP.

P2178**Board Number: B804****Investigating Rho GTPase Pattern Formation During Wound Repair: The Role of RhoGAP1/8.**N. Davenport¹, W. Bement^{1,2,3};¹Graduate Program in Cellular and Molecular Biology, University of Wisconsin-Madison, Madison, WI,²Department of Zoology, University of Wisconsin-Madison, Madison, WI, ³Laboratory of Cell and Molecular Biology, University of Wisconsin-Madison, Madison, WI

The ability to respond to physical damage is an essential and evolutionarily conserved feature of single cells and tissues. The Rho family of GTPases has been shown to coordinate the cytoskeletal rearrangements needed to reestablish the integrity of the plasma membrane and underlying cortex in response to cell damage. The precision with which Rho and Cdc42 are activated and maintained in distinct activity zones throughout the healing process suggests the involvement of multiple Rho GTPase regulators at the wound. To date, a candidate screen for guanine nucleotide exchange factors (GEFs) and GTPase activating proteins (GAPs) involved in regulating Rho GTPase activity at the wound has identified a single wound regulator. By continuing the candidate screen, we have identified two additional GAPs, RhoGAP1 and RhoGAP8, as potential wound regulators. While initially labeling early and recycling endosomes in resting cells, eGFP-tagged RhoGAP1 is recruited between the zones of active Rho and Cdc42 in *Xenopus laevis* oocytes upon wounding. These results have been confirmed by the discovery of endogenous RhoGAP1 at wounds via immunofluorescence microscopy. Targeting of RhoGAP1 to wounds is mediated by its C-terminal region which contains both a GAP domain and a proline-rich region. Reduction of active Rho due to overexpression of untagged RhoGAP1 and alteration of GTPase activity zone position by dominant negative RhoGAP1 supports a hypothesis that RhoGAP1 acts as an enzymatic barrier to promote proper GTPase zone segregation throughout wound repair. We have shown that RhoGAP1 and RhoGAP8 colocalize at single-cell wounds which, in addition to previously reported evidence of their interaction, suggests they may form a complex to regulate Rho GTPase activity. We have also used both RNAi and morpholinos to effectively knockdown proteins in *Xenopus* embryos 24 hours post-fertilization. Knockdown of RhoGAP1 in embryos appears to increase the amount of cytoplasmic F-actin in cells. Further, wounding of *Xenopus* embryos causes 3XeGFP-RhoGAP1 to be displaced from its punctate, junctional localization to become diffuse and cytoplasmic. This displacement appears to follow an intracellular Ca²⁺ increase and precedes increases in both active Rho and F-actin at cell-cell junctions, suggesting RhoGAP1 may regulate a key step in reorganizing the actin cytoskeleton during the multicellular wound response.

P2179**Board Number: B805****A Novel GEF Independent Role of P-Rex1 in GPCR Trafficking.**M.J. Baker¹, E. Maunders¹, H.C. Welch¹;¹The Babraham Institute, Cambridge, United Kingdom

P-Rex proteins are a family of guanine nucleotide exchange factors (GEFs) for the Rho GTPase Rac. They use their GEF function to catalyse the release of GDP from Rac, thus allowing GTP to bind transforming Rac into its active GTP bound conformation. It has been well established that the GEF function of P-Rex1 mediates membrane ruffling and cell migration, as well as reactive oxygen species production. All known cellular responses to P-Rex1 activity are as a result of Rac signalling, often involving actin filament reorganisation. Therefore, there are no known GEF independent roles of P-Rex1. Furthermore, P-Rex proteins are known to be important for tumour growth and metastasis in several types of cancer including prostate, breast and melanoma.

The role of P-Rex family members in trafficking has not been studied in great detail to date, however, there has been work published which suggests that P-Rex1 has roles in vesicle trafficking. This includes evidence to suggest roles for P-Rex1 in trafficking of the GLUT4 transporter¹. We therefore decided to investigate a potential role of P-Rex1 in stimulation dependent GPCR internalisation, in an effort to identify the function of P-Rex1 in trafficking.

During this study we have identified a GEF independent function of P-Rex1 in membrane trafficking. This novel function of P-Rex1 is the first GEF independent function to be identified. Using fixed cell immunofluorescence imaging we show that overexpressed P-Rex1 has an inhibitory effect on the internalisation of a GFP tagged GPCR and that this function of P-Rex1 does not require its GEF activity. We also identify the domains of P-Rex1 that are required for this inhibitory role as part of a first step to deciphering the mechanism behind this exciting and new role of P-Rex1.

1. Balamatsias, D., et al (2011) *Journal of Biological Chemistry* 286, 43229-43240.

P2180**Board Number: B806****Cdc42 Activation is Necessary for Sustained Oscillations of Ca²⁺ and PIP₂ in RBL Mast Cells Stimulated by Antigen.**D.A. Holowka¹, M.M. Wilkes¹, B. Baird¹;¹Chemistry and Chemical Biology, Cornell University, Ithaca, NY

Antigen stimulation of mast cells via FcεRI, the high-affinity receptor for IgE, triggers a signaling cascade that requires Ca²⁺ mobilization for exocytosis of secretory granules during the allergic response. To characterize the role of Rho GTPases in FcεRI signaling, we utilized a mutant RBL cell line, B6A4C1, that is deficient in antigen-stimulated Cdc42 activation important for these processes. B6A4C1 cells exhibit

severely attenuated Ca²⁺ oscillations in response to antigen, and these are restored to wild type RBL-2H3 levels by expression of constitutively active Cdc42 G12V or by a GEF for Cdc42, DOCK7. Ca²⁺ oscillations are not restored when the C-terminal di-arginine motif of active Cdc42 is mutated to di-glutamine, demonstrating a role for these basic residues in this Rho family function. Antigen-stimulated FcεRI endocytosis, which occurs independently of stimulated Ca²⁺ influx, is also defective in B6A4C1 cells, and Cdc42 G12V reconstitutes this response as well. Thus, activation of Cdc42 occurs prior to and is critical for antigen-stimulated pathways leading separately to both Ca²⁺ mobilization and receptor endocytosis. Accounting for these downstream functional consequences, we show that Cdc42 G12V reconstitutes antigen-stimulated oscillations of phosphatidylinositol 4,5-bisphosphate (PIP₂) at the plasma membrane in B6A4C1 cells, pointing to Cdc42 participation in the regulation of stimulated PIP₂ synthesis as a key functional role for this Rho family GTPase in mast cells.

P2181

Board Number: B807

Rac1-dependent activation of Cdc42 during CSF-1 response by macrophages.

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Rac1 and Cdc42, members of the Rho family of small GTPases, are central regulatory hubs of actin cytoskeleton rearrangement required during directed migration. It was previously reported that genetic deletion of Rac1 had no significant effect on macrophage chemotaxis, although a range of abnormalities were observed both in cell morphology and behavior. These results suggest that some compensatory action may occur, possibly by other Rho family members via increased or re-localized activity. To address these potential scenarios we used FRET-based Rho GTPase biosensors, as they can detect dynamic changes both in the levels and localization of Rho GTPase activity. We found that Cdc42 activity was elevated in response to CSF-1 but not significantly localized at F-actin-rich protrusions in a murine macrophage RAW 267.4 cell line, whereas Rac1 activity was highly localized at these same protrusions. However, there was no detectable alteration in Cdc42 activity or localization following reduction of Rac1 levels using shRNA. Surprisingly, shRNA-depletion of Rac1 in these cells produced inhibitory effects on CSF-1 – mediated Cdc42 activation, suggesting a previously unknown potentiation effect by the Rac1 signaling axis. This new regulatory mechanism of Rac1 activating Cdc42 is not due to defects in CSF-1 receptor expression or signaling, since CSF-1 stimulation resulted in similar levels of receptor tyrosine phosphorylation in control versus Rac1-depleted macrophages. We report here a novel signaling mechanism in which Cdc42 activation is dependent on Rac1, and we hypothesize that a Rac1 – activated GEF targeting Cdc42 is involved in the CSF-1 stimulation pathway in macrophages.

P2182**Board Number: B808****AMPylation of Rho GTPases disrupts multiple host signaling processes.**A.R. Woolery¹, X. Yu², J. LaBaer², K. Orth¹;¹Molecular Biology, University of Texas Southwestern Medical Center, Dallas, TX, ²Biodesign Institute, Arizona State University, Tempe, AZ

Rho GTPases are frequent targets of virulence factors as they are keystone signaling molecules in many processes. Modifications of their switch-1 loop are known to be a potent method of disabling the actin cytoskeletal machinery, but Rho GTPases are also important for many other signaling pathways. We attempted to broaden the understanding of the consequences of switch-1 loop modification in downstream signaling using AMPylation by the *Vibrio parahaemolyticus* type three secreted effector VopS. Using infection models, biochemistry and versatile self-assembled microarrays, several novel effects of Rho GTPase AMPylation were identified. We found that multiple signaling interactions of GTPases were inhibited, including NFκB, MAP kinase, the Inhibitor of Apoptosis proteins (IAP) and the phagocytic NADPH oxidase system (NOX2.) Phosphorylation of IκB^α and JNK kinase was inhibited in a VopS-dependent manner during infection with *Vibrio parahaemolyticus*. VopS also sequestered p65 in the cytosol during infection, while removal of VopS allowed robust translocation. AMPylation also prevented the generation of superoxide by the phagocytic NADPH oxidase complex. Furthermore, the interaction of GTPases with the E3 ubiquitin ligases cIAP1 and XIAP was hindered, leading to decreased degradation of Rac and RhoA during infection. Finally, we screened for novel Rac1 interactions using a nucleic acid programmable protein array (NAPPA) and discovered that Rac1 binds to the protein C1QA, a protein known to promote immune signaling in the cytosol. Interestingly, this interaction was disrupted by AMPylation. In addition to these findings, we also adapted NAPPA to use fluorescent probes to screen for novel substrates of protein transferases, including AMPylation. We conclude that modification of the switch-1 loop by VopS and other toxic proteins is a multifaceted virulence mechanism that counters several host immunity strategies.

P2183**Board Number: B809****Rac1 orchestrates transcriptional and post-transcriptional regulation of matrix remodelling during fibroblast-mediated matrix contraction.**H. Li¹, V. Tovell¹, D. Paull¹, D. Simpson², P.T. Khaw¹, M. Bailly¹;¹Department of Cell Biology, UCL Institute of Ophthalmology, London, United Kingdom, ²Centre for Experimental Medicine, Queen's University, Belfast, United Kingdom

Fibrosis is a major cause of morbidity in humans, with over 40% of all death in the developed world attributable to some form of fibrotic disease. In the eye alone, tissue contraction and scarring are involved in the pathogenesis or failure of treatment of virtually all major blinding diseases. However, the molecular mechanisms underlying fibrosis and scarring are still poorly understood. We previously

identified Rac1 as one of the major player underlying fibroblast-mediated tissue contraction, and showed that an early transient treatment with Rac1 inhibitor NSC23766 is sufficient to block long-term matrix remodelling and tissue contraction *in vitro* and *ex-vivo*. We have now performed a genome wide microarray analysis in primary human conjunctival fibroblasts during contraction *in vitro* with/without treatment with NSC23766. We have found that the cells undergo dramatic shift in gene expression during the early stages of contraction, akin to a wound activation profile, with over 3000 genes changing expression from day 0 to day3, in which a specific gene profile including matrix metalloproteinases (MMP) 1, 3 and 10, inflammatory molecules and cytokines are significantly up-regulated. This “activation” profile recedes when the contraction slows down at day5, with most of the genes changing back in the opposite direction from day3 to day 5, and the gene profile of later contraction is linked to long-term fibrosis with gene expressions found in common with a few human ocular fibrotic diseases. Transient treatment with the Rac1 inhibitor resulted in an almost complete abrogation of the activation phase, with the cells assuming a day5 resting cell profile. In addition, we showed that whilst Rac1 inhibition did not affect the mRNA expression of most MMPs upregulated during contraction, it lead to a significant reduction in overall MMP activity, in accordance with the previously observed reduction in matrix degradation. This suggests that blocking Rac1 activation during the early stages of contraction is sufficient to fully prevent the cells from entering the contractile pathway as a whole, as Rac1 appears to mastermind a complete wound healing response by acting both at the transcriptional and post-transcriptional level.

P2184

Board Number: B810

Rac3 promotes invadopodia maturation and matrix degradation.

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Metastasis is responsible for ninety percent of all cancer related deaths. One of the first steps in the metastatic cascade is the local invasion of tumor cells into the surrounding tissue. Invadopodia are actin-based protrusions that mediate the extracellular matrix degradation necessary for the invasion of tumor cells. Therefore deciphering the mechanisms of invadopodia regulation is critical to furthering our understanding of cancer cell dissemination. A number of studies have implicated the small GTPase Rac3 in tumor cell invasion but its mechanism of action has not yet been elucidated. Despite the similarity of Rac3 and the better-studied Rac1, there is also evidence to suggest that these isoforms have different functional roles. Rac1 has recently been shown to regulate the disassembly of invadopodia. We now show that, in direct contrast to Rac1, Rac3 regulates the maturation of invadopodia in two breast cancer cell lines, MtlN3 and MDA-MB-231. As Rac1 and Rac3 share 93% sequence homology, traditional approaches are not optimally suited to precisely define the spatial and temporal dynamics of Rac3 activation at invadopodia. Thus we have generated and validated a novel single-chain FRET-based Rac3 biosensor. Preliminary results suggest that there is a transient activation of Rac3 at invadopodia. Taken

together our data suggests that Rac3 functions as a molecular switch that regulates the transition of invadopodia from precursor to mature structure.

P2185

Board Number: B811

Cell invasion through basement membrane requires multiple GTPases and is initiated by CDC-42 directed invadopodia formation.

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Basement membrane (BM) is a thin, dense, sheet-like extracellular matrix that underlies epithelia, endothelia, and enwraps muscle and adipose tissues. Cell invasion through BM occurs during development and physiological processes, and inappropriately in cancer metastasis. Extrinsic signaling cues play key roles in inducing invasion, but how these signals regulate the cellular machinery and behaviors that promote invasion remains poorly understood. A key barrier in understanding how extrinsic cues direct invasion is the challenge of experimentally examining the dynamic interactions between invasive cells, BM, and the tissue being invaded *in vivo*. Anchor cell (AC) invasion in *C. elegans* is an experimentally tractable model to uncover mechanisms controlling cell invasion *in vivo*. To identify new genes required for AC invasion, we performed a sensitized genome-wide RNAi screen and found that the Rho GTPase *cdc-42* promotes AC invasion. In response to extracellular signals, CDC-42 can be activated to regulate processes related to cell invasion, such as cell polarity and cytoskeletal organization. Here we show that animals null for *cdc-42* have defects in AC invasion and display a highly penetrant delay in initial breach of the BM. An unknown cue secreted from the vulval precursor cells controls the timing and initiation of invasion, but the molecular pathways downstream of this cue have not been identified. Using site-of-action and genetic analyses, we show that CDC-42 functions in the AC downstream the vulval cue. While *cdc-42* has been identified as a regulator of cancer invasion *in vitro* how CDC-42 is localized, activated, and functions in BM breaching is unknown. A genetically encoded biosensor for active CDC-42 shows that CDC-42 is strongly activated at the site of BM breach, and that active CDC-42 colocalizes with highly dynamic F-actin structures called invadopodia, which are responsible BM breaching in AC invasion. RNAi knockdown of *cdc-42* resulted in a significant reduction in invadopodia number. These results suggest *cdc-42* is downstream of the vulval cue and initiates BM breaching by promoting invadopodia. Interestingly, an additional extrinsic signaling pathway functions in the AC involving the netrin receptor (*unc-40*) and its effectors, the Rac GTPases *ced-10* and *mig-2*. UNC-40 and the Rac GTPases direct formation of a large invasive protrusion after the vulval cue initiates BM breach via CDC-42 and invadopodia. Altogether, our data suggest that multiple, independent cues are required to direct cell invasion through BM (a coincidence system). We speculate that such systems exist to precisely control the site and timing of cell invasion—an essential but potentially destructive biological process.

Signaling Networks Governing Cell Migration

P2186

Board Number: B813

A Cell Life under Confinement: how immune cells manage to move, squeeze and survive.

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The quest to understand how the mechanical and geometrical environment of cells impacts their behavior and fate has been a major force driving the recent development of new technologies in cell biology research. Despite rapid advances in this field, many challenges remain in order to bridge the gap between the classical and simple cell culture plate and the biological reality of actual tissues. In tissues, cells have their physical space constrained by neighboring cells and extracellular matrix. In the recent years, we have developed simple and versatile devices to precisely and dynamically control this confinement parameter in cultured cells. I will present results we obtained on the effect of confinement on cell migration focusing on two questions: how modulating confinement and adhesion of slow mesenchymal migrating cells can make them switch to a fast amoeboid like migration behavior and how cells can squeeze their nucleus when migrating through small gaps. I will conclude presenting intriguing results showing that large deformations of migrating cells induce strong damages to their nucleus raising the question of how immune cells such as dendritic cells can combine high motility and long term survival.

P2187

Board Number: B814

TRIM9 regulates DCC trafficking and FAK activity during developmental axonal plasma membrane expansion.

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During neuronal development, growth cones at the tips of axons branch and extend toward postsynaptic targets. This involves dramatic expansion of the plasma membrane and requires regulated delivery of new plasma membrane material. The axon guidance cue Netrin-1 and its receptor DCC promote exocytosis, attractive growth cone turning and axon branching at least partially through the activation of the non-receptor tyrosine kinase FAK. We identified the E3 ubiquitin ligase TRIM9 as a novel regulator of exocytosis, axon branching and axon guidance. We show that TRIM9 directly binds the cytoplasmic tail of DCC within the same domain bound by FAK. Using neurons isolated from

embryonic mouse cortex, we demonstrate that genetic loss of TRIM9 disrupts DCC trafficking, activation of FAK, the frequency of exocytic vesicle fusion, and the density of axon branching. TIRF microscopy and surface biotinylation assays indicate that following Netrin-1 stimulation, DCC clusters and accumulates in the plasma membrane and is subsequently endocytosed and degraded. In TRIM9^{-/-} cortical neurons, DCC fails to cluster in the plasma membrane and does not get degraded following Netrin-1 stimulation. Whereas clustering is rescued by expression of GFP-TRIM9, structure function studies indicate that the ubiquitin ligase domain of TRIM9 is not required for clustering, but is necessary for Netrin response. Although total DCC protein is reduced in TRIM9^{-/-} neurons, a higher proportion of DCC localizes at the cell surface. These defects in DCC trafficking are associated with hyperphosphorylation of FAK at Y397, an increased exocytic frequency and exuberant axon branching in TRIM9^{-/-} neurons. Pharmacological inhibition of FAK rescues the aberrant exocytosis and axon branching in TRIM9^{-/-} neurons and blocks Netrin-dependent increases in exocytosis and branching in wildtype neurons. Interestingly, the exuberant branching of TRIM9^{-/-} axons in vitro was also observed in vivo within the corpus callosum and correlated with increased callosal thickness. We have crossed TRIM9^{-/-} and FRNKlox mice to determine if inhibition of FAK blocks the exuberant axon branching and corpus callosum thickening defects found in vivo. Results from cortical explant assays indicate TRIM9 also functions during axon outgrowth toward an asymmetric Netrin source. We are using a membrane-targeted GFP driven by the Tau promoter to define the axonal projection defects in TRIM9^{-/-} mice in vivo. We conclude that TRIM9 regulates DCC trafficking and FAK activation to control vesicle fusion and plasma membrane expansion during Netrin-dependent axon responses.

P2188

Board Number: B815

Characterizing new genes regulating cell-substrate adhesion to discover novel regulatory mechanisms of cell motility.

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The model organism Dictyostelium has greatly facilitated our understanding of the signal transduction and cytoskeletal pathways that govern cell motility. Cell-substrate adhesion is a target of many chemotaxis signaling events and it can be used to screen for cells that have defects in cell migration. In fact, cells lacking PTEN, a negative regulator of cellular extensions, adhere strongly to the surface. This leads to reasoning that other regulators of migration would also effect adhesion, a screening method was devised and isolated overly adherent mutants from a pool of mutagenized cells. Restriction enzyme mediated insertion (REMI) mutagenized cells, comprising more than 50000 insertions, yielded about 100 mutated cell lines with the desired phenotypes. The mutation sites in 20 of the strains have been mapped and many of the phenotypes are similar to those of PTEN knockout cells. The extent of increased adhesion, cell motility, directed migration, cell shape, and new filamentous actin at the periphery are all parameters that have been examined in these new overly adhesive cell lines. The

degree in which these parameters have been effected and the correlations between these changes is providing novel insights into the networks controlling cell motility. Many of these genes have human homologs with unknown functions. Therefore, the future study of this new group of regulators of adhesion and motility genes in Dictyostelium will not only advance the knowledge of cell migration in amoeboid cells but elucidate the functions of novel human genes with potential disease relevance.

P2189

Board Number: B816

Synthetic manipulation of PIP2 levels and PIP2-associated chemotactic signaling dissection in Dictyostelium.

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PIP2 is linked to many key chemotactic players, but our understanding of its roles in chemotactic signaling is very limited due to technical difficulties. The idea that the localized decrease of PIP2 level initiates chemotactic signaling events has been around for years, but has never been tested in a direct manner with traditional methods. In order to illuminate the roles of PIP2 and further explore the features of the chemotactic signaling network, I used the chemically-induced dimerization (CID) system, which allows the rapid inducible translocation of cellular proteins, to synthetically manipulate PIP2 level and dissect the signaling events. I have successfully accommodated CID in Dictyostelium, an excellent model system to study eukaryotic chemotaxis. In my current data, after I synthetically recruited the PIP2-specific 5-phosphatase Inp54p to the plasma membrane, the PIP2 biosensor PHplc δ fell off the membrane and the cells robustly oscillated between a spreading and a crunching morphology. Cells with the spreading morphology, but not the other one, may have further lowered PIP2 levels indicated by PTEN localization, clearly activated chemotactic signaling events as shown by Ras and PIP3 biosensors, and highly elevated F-actin along the periphery. Further, cells still carried out this response when PIP3 production was diminished by the PI3K inhibitor LY. Based on these results, we propose that PIP2 serves as an inhibitory role on Ras; when PIP2 levels are decreased, the chemotactic signaling network containing PKBs is activated following Ras activation. Also based on previous evidence, we propose that PI5K activation is downstream of PKBs activation, which brings up PIP2 levels to a certain degree and leads to the crunching morphology after cell spreading following the synthetic Inp54p recruitment. I will further use this system to dissect the PIP2-initiated signaling and feedback loops, as well as their roles in cell polarity and chemotaxis. Together the results of these experiments should shed light on the unsolved roles of PIP2 in chemotaxis and contribute to our further understanding of the chemotactic signaling network.

P2190**Board Number: B817****The adaptor proteins Crk and CrkL regulate T cell adhesion and selectively promote migration to sites of inflammation.**Y. Huang¹, M. Karimi², T. Park¹, N. Roy¹, T. Curran¹, T. Kambayashi², J.K. Burkhardt¹;¹Department of Pathology and Laboratory Medicine, The Children's Hospital of Philadelphia Research Institute, Philadelphia, PA, ²Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA

Effector T cell migration into inflamed sites greatly contributes to local inflammation and disease severity in inflammatory diseases, including GVHD. T cell migration into such sites depends heavily on regulated adhesion and migration, but the signaling pathways that coordinate these functions downstream of chemokine receptors are largely unknown. Using conditional knockout mice, we have found that T cells lacking the adapter proteins Crk and CrkL exhibit reduced integrin-dependent adhesion, chemotaxis, and diapedesis across an endothelium. Our studies reveal significant functional redundancy of the two closely related proteins. We show that Crk proteins coordinate with C3G and CasL to activate the integrin regulatory GTPase Rap1. Importantly, Crk proteins are required for effector T cell trafficking into sites of inflammation, but not for migration to lymphoid organs. This unique differential migration of Crk/CrkL-deficient T cells has important therapeutic implications, since T cells lacking Crk proteins can carry out graft-versus-leukemia responses with minimal graft-versus-host disease. Our studies show that Crk family adaptor proteins selectively regulate T cell adhesion and migration at effector sites, and identify these proteins as important novel therapeutic targets.

P2191**Board Number: B818****Coagulation factor IX regulates cell migration and adhesion in vitro.**A. Mamiya¹, H. Kitano¹, T. Ishikawa¹, Y. Fujiwara¹, M. Yoshida¹, . Kokubun¹, C. Hidai¹;¹Nihon Univ Sch Med, Tokyo, Japan

Introduction : Coagulation factor IX (F9) is thought to circulate in the blood as an inactive zymogen before being activated in the coagulation process. The effect of F9 on cells is poorly understood. This study aimed to evaluate the effects of intact F9 and its cleavage fragments on cell behavior. Materials & Methods : A431 cells (derived from human squamous cell carcinoma), Pro5 cells (derived from mouse embryonic endothelial cells), Cos7 cells, and human umbilical vein endothelial cells (HUVEC) were utilized in this study. The effects of F9 and its cleavage fragments on cell behavior were investigated in several types of experiments, including wound-healing assays and modified Boyden chamber assays. To investigate intercellular or cell-matrix adhesion, immunohistochemistry was employed. Results : The effect of F9 depended on its processing; full-length F9 suppressed cell migration, increased adhesion to matrix, and enhanced intercellular adhesion. In contrast, activated F9 enhanced cell migration,

suppressed adhesion to matrix, and inhibited intercellular adhesion. the effect of F9 appears to be nonspecific with regards to cell type, as the effect was demonstrated in cells of diverse origin (i.e., epithelial [A431], endothelial [pro5], and mesenchymal [cos7]). An activation peptide that is removed during the coagulation process was found to be responsible for the activity of full-length F9, and the activity of activated F9 was localized to an EGF domain of the F9 light chain. Our data indicated that the activation peptide inhibited the activity of the EGF domain on cells. Conclusion: We found that in addition to its role in the coagulation process, F9 has several novel effects on cells. Full-length F9 has a sedative effect on cells, which is counteracted by activated F9 in vitro. Thus, F9 may play roles before, during, and after the coagulation process.

P2192

Board Number: B819

Dose-dependent histomorphometric and ultrastructural changes in the testis of adult male Japanese quails (*Coturnix coturnix japonica*) exposed to di-n-butyl phthalate (DBP), in vivo.

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We have investigated the dose-response effects DBP on histo-morphometric parameters and ultrastructure of the seminiferous tubules of adult male Japanese quails (*C. japonica*). A total of ninety (90) pre-sexed, 10-week old male quail birds were randomly divided into six experimental groups: The control group (n=15) received a dose of 1 mL/kg of corn-oil (control), or intragastric daily dose of 1, 10, 50, 200, 400 mg DBP/kg/bw (dissolved in corn oil), for a period of 30-day. At study termination, the testes excised from both control and treatment groups and were further evaluated using a computer-aided histometric and ultra-structural (TEM) techniques. Our results showed a generalized significant decrease in the diameter and epithelium thickness (height) of the seminiferous tubules occurring predominantly in the high dose (200mg- and 400mg) groups as compared to median (50mg) or low dose (1mg- and 10mg) groups. Ultra-structurally, a dose-dependent degenerative changes in earlier germ cell series, and being significant at the high dose level, were evident. Necrotic round spermatids were prominent in sub luminal part of the seminiferous tubules amidst apparently normal elongated spermatids. A few spermatogonia were necrotic, and a few empty spaces, probably vacated by necrotic early spermatocytes or spermatogonia, were encountered. Few spermatogonia showed degenerative changes with morphological characteristics of apoptosis-- including shrinkage of cytoplasm and increased nuclear blebbing, occurring mainly in high doses (i.e. 200- and 400mg DBP) groups. Sertoli cells appeared foamy in consistency because they are laden with lipid droplets. Dense bodies were also conspicuous and very importantly, the mitochondria were swollen and showed a disrupted matrix. In

addition, Leydig cells were few and showed numerous lipid droplets (scattered throughout the cytoplasm), multivesicular bodies and giant whorl-like smooth endoplasmic reticulum. In conclusion, the present data indicate that DBP(pubertal) exposure produced dose- and parameter-dependent, histometric and sub-cellular alterations in the seminiferous tubules, thereby affecting the adult spermatogenesis. It is evident that DBP pronounced male reproductive toxicities in testes might likely be due to its anti-androgenic effect on reproduction, resulting from increased damaged to earlier spermatogenic series, Sertoli and Leydig cells. The potential negative implication on the reproductive biology, particularly in those avian species, that are vulnerable to EDCs are further discussed.

P2193

Board Number: B820

High glucose reduces migration and viability of human periodontal ligament cells.

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Background: Impaired oral wound healing and increased incidence of periodontal diseases are complications of Diabetes Mellitus (DM). DM impairs the migration of dermal fibroblasts, contributing to deficient healing, and some microRNAs (miRs), such as miR-221 and miR-222, are affected by DM and might be involved in cellular functions. **Aim:** to evaluate the effects of the *in vitro* exposure of human periodontal ligament (PLC) cells to high glucose on growth, death, migration, actin cytoskeleton and the expression of miR-221 and miR-222. **Methods:** cells were obtained from premolar teeth of 3 young humans under orthodontic treatment, and cultured under low (LG, 5mM) or high (HG, 25mM) glucose for 7 days before the assays. **Results:** Cell number was reduced by 33% in HG cells, compared to LG, mostly due to a 3x increased in cell death (TUNEL assay). Cell adhesion on fibronectin was reduced by 40% after 20 minutes of plating, and by 30% after 1 hour. Cell spreading after 1h was reduced by 35%. HG cell migration (transwell assay) was reduced by 54%. HG cells showed increased circularity when compared to LG (> 0.5 vs. 0.2, respectively). The expression of miR-221 and miR-222 was increased by 2x in HG cells. **Conclusion:** High glucose negatively affects PLC, reducing migration and viability, thus potentially affecting the progression of periodontal disease and healing after periodontitis treatment, as well as the response to orthodontic treatment. miR-221 and miR-222 were upregulated by HG in those cells and might be involved in some of these events. **Support:** FAPESP, CAPES, PRP-USP (NAPmIR).

P2194**Board Number: B821****High Glucose concentration reduces cellular contractility and tension forces in NIH3T3 fibroblasts, contributing to the impaired migration in 2D and 3D substrates.**M.E. Almeida¹, M. Badoual², M. Lamers³, A.R. Horwitz⁴, M.F. Santos⁵;¹Cell and Development Biology, University of Sao Paulo, Sao Paulo, PA, ²Laboratoire IMNC, Univ. Paris-Sud, Orsay, PA, ³Morphological Sciences, Universidade Federal Do Rio Grande Do Sul, Porto Alegre- RS, PA, ⁴Cell Biology, University of Virginia, Charlottesville, VA, ⁵Cell and development Biology, Univ Sao Paulo, Sao Paulo, Brazil

Background: Long-term complications of Diabetes Mellitus include impaired wound healing and fibroblast migration and extracellular matrix (ECM) remodeling are essential for this process. We have previously shown that fibroblasts migration is reduced under high glucose conditions as well as the maturation of cellular adhesions to the ECM. Another feature of those cells is the long rear and less stress fibers, which suggest impaired myosin II activity. During migration, the contraction of the cell body generates tension on the substrate through cell adhesions, which is important for adhesion maturation. Aim: To study the effects of a high glucose concentration on fibroblast contractility and its potential contribution to the impairment of migration. Methods: NIH3T3 fibroblasts were exposed to physiological (5mM, LG) or high (25mM, HG) glucose concentrations and submitted to different analyses on two-dimensional (2D) fibronectin and three-dimensional (3D) collagen/fibronectin substrates. F-actin arrangement and the expression and distribution of the myosin regulatory light chain (MRLC), myosin isoforms IIA (MIIA) and IIB (MIIB) were studied by staining with phalloidin, western blotting and immunocytochemistry, respectively. Traction force microscopy (TFM) assays were employed to study tension exerted on the substrate. Results: Cells exposed to HG showed reduced migration velocity (about 40-60%) and larger lamellipodia on 2D assays. In 3D substrates, HG cells showed extensive branching projections at the front. TFM experiments showed lower tension exerted by HG cells on the adhesions to the substrate. HG did not affect the total amount of MRLC, MIIA and MIIB proteins. MIIB distribution, however, was changed. In cells cultured under low glucose MIIB was mostly present in the cell body and rear, whereas in HG cells it was spread throughout the cell, including the lamellipodia. MIIA distribution, on the other hand, was not changed. Conclusion: fibroblasts under high glucose show impaired contractility, which leads to a reduced tension on the substrate through cellular adhesions. This impairment may be involved in the deficient migration of fibroblasts in diabetic patients. Support: FAPESP, CAPES.

P2195**Board Number: B822****Free β -subunit of human chorionic gonadotropin stimulates choriocarcinoma cell invasion independent of LH/hCG receptor.**C. Lee¹, P.C. Chiu¹, L. Hautala², W.S. Yeung¹, K.M. Koli³, U. Stenman², H. Koistinen²;¹Department of Obstetrics and Gynecology, The University of Hong Kong, Queen Mary Hospital, Hong Kong, Hong Kong, ²Department of Clinical Chemistry, University of Helsinki, Helsinki, Finland, ³Research Programs Unit, Translational Cancer Biology, University of Helsinki, Helsinki, Finland

Human chorionic gonadotropin (hCG) is a heterodimer consisting of an α - and a β -subunit (hCG α and hCG β) that are non-covalently linked. Intact hCG, but not free subunits, bind to the LH/hCG receptor in the corpus luteum stimulating its progesterone production, which is essential for successful pregnancy. hCG has been found to stimulate trophoblast invasion in an autocrine fashion. In pregnancy, trophoblast invasion is a tightly controlled process, disturbances in which may lead to pregnancy complications. In addition to placenta, several cancers have been shown to express hCG and hCG β . Most trophoblastic tumors of placental and germ cell origin produce hCG, while many non-trophoblastic tumors, including bladder, renal and gastrointestinal cancers, often produce hCG β . The stimulatory activity on trophoblast invasion has been ascribed especially to a so-called hyperglycosylated form of hCG (hCG-h) expressed in early pregnancy. hCG-h is also a major form of hCG produced by choriocarcinoma and testicular cancers, although tumor-derived hCG-h is often glycosylated differently than that produced by the placenta. We compared different forms of hCG in their stimulatory activities on the invasion of JEG-3 choriocarcinoma cells. Various forms of hCG and hCG β were purified from urine of a patient with nonseminomatous testicular cancer, one with invasive molar disease, and a pregnant women. Both hCG and hCG β , and their hyperglycosylated forms stimulated the invasion of JEG-3 cells to a similar extent. Down-regulation of the LH/hCG receptor by RNA-interference did not significantly reduce the effect of hCG β and hCG on cell invasion. Increased invasion was associated with increased activity of MMP-2, MMP-9 and uPA in conditioned culture medium from JEG-3 cells. Unlike suggested by some previous studies, highly purified hCG or hCG β , or their hyperglycosylated forms, did not activate TGF β -receptor or induce phosphorylation of ERK1/2. Our findings suggest that hCG and hCG β stimulate the invasion of choriocarcinoma cells by an unknown mechanism, that is independent of the classical LH/hCG-receptor. These results may explain the association of increased serum hCG and hCG β with adverse prognosis both in trophoblastic and non-trophoblastic tumors.

P2196**Board Number: B823****The role of an R-Ras/ARF6 signaling module in adhesion-induced epithelial spreading and migration.**J.C. Salem¹, L.C. Santy²;¹Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA, ²Department of Biochemistry, Microbiology and Biochemistry, Pennsylvania State University, University Park, PA

Overexpression of ARF-GEF cytohesin-2/ARNO causes immobile epithelial cells to become highly migratory and scatter away from neighboring cells. Through robust activation of ARF6 and assembly of a multi-protein complex, cytohesin-2/ARNO behaves as both an ARF-GEF and a scaffold to promote activation of small GTPase Rac. R-Ras is a small GTPase that specializes in enhancing cell spreading and promoting integrin activation. It was recently shown that small GTPase R-Ras signals upstream of cytohesin-2/ARNO to promote adhesion-induced migration. Given that both R-Ras and cytohesin-2/ARNO function in a similar pathway, we wondered if ARF-activation via cytohesin-2/ARNO could direct R-Ras traffic, and if so, through which intracellular compartment. Therefore, transfected HeLa cells were plated on fibronectin, and imaged using fixed and live immunofluorescence. We demonstrate that overexpressed R-Ras traffics through a tubular EHD1 recycling endosome compartment. Destroying the GEF activity of cytohesin-2/ARNO (E156K) traps R-Ras in EHD1 recycling endosomes. In contrast, phorbol 12-myristate 13-acetate (PMA), a cytohesin-2/ARNO stimulator, promotes dissolution of EHD1 tubular endosomes containing R-Ras. Both R-Ras and ARNO each promote cell spreading, as constitutively active (38V) R-Ras can only partially rescue cell spreading in cells co-expressing E156K ARNO. Finally, we provide evidence suggesting α 5-integrin is also trapped in an EHD1 compartment upon E156K-ARNO expression. Given that cytohesin-2/ARNO is required for β 1-integrin recycling and migration on fibronectin, we propose that R-Ras effects on integrin activation, ruffling, and spreading are highly governed by cytohesin-2/ARNO-induced ARF6 activation.

P2197**Board Number: B824****A novel mechanism of cell locomotion regulation by LOSK/SLK kinase.**A.I. Fokin¹, T.S. Klementyeva², E.S. Nadezhdina^{1,2}, A.V. Burakov¹;¹A.N.Belozersky Institute for Physico-Chemical Biology, Lomonosov Moscow State University, Moscow, Russia, ²Institute of Protein Research of Russian Academy of Sciences, Moscow, Russia

Cell locomotion is a complex process which requires both actin and microtubule cytoskeleton involvement. Actomyosin component of the cytoskeleton generates force for movement. Microtubules are assumed to induce cell polarization and maintain direct movement. SLK is a serine/threonine-protein kinase associated with microtubules and centrosome, originally described as LOSK (Long Ste20-Like). Inhibition of its activity results in disorganization of microtubule system and reduction of Golgi polarization in moving cells (Burakov et al., 2008, Zhapparova et al., 2013). Hence they demonstrate

decreased ability to directional movement.

It is known that LOSK phosphorylates a series of cytoskeletal proteins such as RhoA (Guilluy et al., 2008) and p150Glued subunit of dynactin (Zhapparova et al., 2013) which are essential for cell locomotion. To test the role of RhoA and p150Glued phosphorylation by LOSK we performed transfection of Vero fibroblast-like cells with dominant negative LOSK[K63R-ΔT], dominant negative and constitutively active RhoA, phospho-mimic and non-phosphorylatable mutants of RhoA and p150Glued at LOSK phosphorylation site. Moreover we co-transfected cells with RhoA and p150Glued constructions described above against LOSK inhibition by LOSK[K63R-ΔT]. Also we used inhibitor of downstream RhoA effector ROCK kinase. Standard wound scratching assay and ImageJ plugin Manual tracking were applied to specify parameters of cell movement. Besides we examined in details the structure of microtubule system and polarization of Golgi in these cells. We estimated that the regulation of cell motility and the microtubule ordering by LOSK occurs independently in two different signaling pathways. It is turned out that cell motility impaired by LOSK inhibition could be restored, using phospho-mimic (inactivated) RhoA as well as ROCK inhibitor; also it partially rescued Golgi polarization despite the fact that the microtubules remains disordered. On the other hand, the p150Glued construct which could not effectively rescue cell motility, successfully restores the microtubule system and Golgi polarization. Thus, it was first shown in this study that LOSK-dependent microtubule disordering does not affect the cell motility and regulation of cell migration is accomplished through another LOSK-dependent signaling pathway. This work is financially supported by the Russian Foundation for Basic Research (grants 12-04-33178 mol-a-ved and 14-04-01729a).

P2198

Board Number: B825

Analysis of Diatom Motility in Multi-species Assemblages.

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Diatoms are unicellular algae, present in almost all freshwater and marine aquatic environments, known for their characteristic hardened silica cell walls. Diatoms live within complex multi-species algal communities, and provide a large percentage of earth's primary production and oxygen formation. Investigating the environmental effects on cell behavior, such as the light-regulated gliding motility of pennate diatoms that is crucial for their ecological success, is therefore critically important to understanding their overall ecological sustainability. Previous studies in our lab suggested that the presence of multiple diatom species alters the cells' adhesive ability relative to cells from unialgal cultures (*Diatom Res.* **18** :225). In order to determine if the presence of other species also affects cell motility, we analyzed the motile responses of three diatom species (*Craticula cuspidata*, *Pinnularia viridis*, and *Stauroneis phoenicenteron*) to high intensity irradiations of blue (470nm), or red (650nm) light at their leading or trailing ends, in the presence or absence of one of the other diatom species. Our analysis of the response times of light-stimulated direction changes in these cells suggests that under some conditions there are significant inter-species effects on diatom motility, which may relate to their

niche partitioning. For example, while *Craticula* cells show little change in their response times in the presence of other species, *Stauroneis* cells show a strong repression in response times for blue light exposures of their leading ends in the presence of *Craticula* cells (36 ± 3 sec for *Stauroneis* alone; 88 ± 9 sec for *Stauroneis* in the presence of *Craticula*). *Stauroneis* cells show similar repression to red light exposures of the leading end (96 ± 10 sec for *Stauroneis* alone; 148 ± 13 sec for *Stauroneis* in the presence of *Craticula*). In contrast, *Pinnularia* cells show decreased response times for red-light leading end exposures in the presence of *Craticula* (163 ± 20 sec for *Pinnularia* alone; 92 ± 21 sec for *Pinnularia* in the presence of *Craticula*). We have also analyzed the effect of multiple sequential trailing end cell irradiations to determine the degree to which these exposures repress subsequent direction changes at the leading end. With blue irradiations of the trailing end, *Craticula* and *Stauroneis* show a strong dose-dependent effect that indicates the repression lasts 30-90 sec, while *Pinnularia* shows a longer lasting repression that remains for more than 2 min. Red irradiations of the trailing end of *Stauroneis* cells show an effect that lasts 90-120 sec. This work was supported by grants through the DePaul College of Science and Health, the DePaul University Research Council, and equipment purchased previously through NSF Grant IBN-9982897.

P2199

Board Number: B826

Syndecan-4 regulates Eph receptor-mediated fibroblast clustering during wound repair.

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Fibroblast migration into a wound bed is essential for wound contraction and healing. Migration relies on cell-matrix and cell-cell contacts, both of which are regulated by transmembrane receptors that are differentially regulated during wound healing. The expression of the fibronectin receptor syndecan-4 is increased upon wounding, which in fibroblasts promotes migration through activation of small GTPases and the recycling of integrin $\alpha 5\beta 1$. Members of the Eph family of receptor tyrosine kinases, which regulate cell-cell contacts, are also differentially regulated in fibroblasts upon wounding. However, little is known about the relationship between these receptors and how this might regulate the balance between cell-cell and cell-matrix contacts.

We demonstrate an inverse relationship between syndecan-4 and EphA2, which mediates cell-cell repulsion. EphA2 expression is elevated in syndecan-4 knockout mouse embryonic fibroblasts through a Src family kinase-dependent mechanism. Elevated EphA2 expression increases sensitivity to the EphA2 ligand ephrin-A1 and increases the rate and frequency of cell-cell repulsion when compared to wild type. Importantly, our findings are translated *in vivo*; EphA2 expression is increased in syndecan-4 knockout mouse wounds compared to wild type. This may contribute to the healing defect previously observed in syndecan-4-null mice¹.

¹Echtermeyer, F. et al. Delayed wound repair and impaired angiogenesis in mice lacking syndecan-4. *J. Clin. Invest.* 107, R9-R14 (2001).

P2200

Board Number: B827

Na⁺/Ca²⁺ exchanger 1 regulates Ca²⁺ dependent ERK signaling and migration.

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The sodium pump, Na⁺/K⁺-ATPase, contains a catalytic α -subunit (Na,K- α) and a regulatory β -subunit (Na,K- β). These proteins regulate ion homeostasis, in addition to maintaining epithelial polarity and structural integrity. The activity of Na⁺/K⁺-ATPase is closely coupled with another transporter, Na⁺/Ca²⁺ exchanger 1 (NCX1). The efflux of 3 Na⁺ by Na,K-ATPase provides the gradient for NCX1, which enables the influx of 3 Na⁺ and efflux of 1 Ca²⁺. Earlier studies from our lab have shown that mice with heart-specific ablation (KO) of Na,K- β were insensitive to ouabain-induced cardiac contractility with reduced NCX1 expression, and no change in other Ca²⁺ handling proteins (Barwe et al., *J. Mol Cell Cardiol*, 2009). In addition we show that Na,K- β co-localizes with NCX1 and confirm their association by immunoprecipitation. These data support the hypothesis that Na,K- β acts as a chaperone in regulating the membrane expression and localization of not only Na,K- α , but also NCX1. Other studies have shown a similar role for sodium transporters such as Na,K,2Cl transporter (Carmosino et al., *J. Hypertension*, 2014). To understand the cause and consequences of this reduced NCX1 expression, we utilized Madin Darby Canine Kidney (MDCK) cells with Na,K- β knockdown (β -KD). β -KD cells express reduced levels of NCX1, accompanied by a 2-fold increase in free intracellular calcium concentration, consistent with the fact that NCX1 is the major calcium extrusion mechanism in MDCK cells. A corresponding increase in the rate of migration was observed using wound healing assay. Replenishment of Na,K- β in β -KD (β -KD/WT-R) cells restored NCX1 protein at the cell surface, reduced the intracellular calcium levels and migration rate similar to that in parental MDCK cells. The Ca²⁺ chelator BAPTA suppressed migration in β -KD cells, indicating that the increased migration was calcium-dependent, thus implying a role for NCX1 in the regulation of migration. MDCK cells treated with NCX1 inhibitor, KB-R7943, showed an increase in cell migration similar to β -KD cells, confirming the role of NCX1 activity in regulating cell migration. To delineate the signaling pathways in the regulation of cell migration by NCX1, we used specific inhibitors of PI3-kinase, Akt and MEK1/2, since β -KD cells display PI3-kinase dependent ERK1/2 and Akt activation (Barwe et al., *J Cell Sci*, 2012). Our data revealed that migration induced by reduction of NCX1 in β -KD cells or NCX1 inhibition is dependent on PI3-kinase and ERK1/2 (direct downstream target of MEK1/2), but is Akt-independent.

P2201**Board Number: B828****Activation of Rac by Asef2 Promotes Myosin II-Dependent Contractility to Inhibit Cell Migration in both 2D and 3D Environments.**L. Jean¹, D. Majumdar¹, M. Shi¹, L. Yang², Y. Gao², J. Broussard¹, L. Hinkle¹, B. Brewer², D. Li^{2,3}, D. Webb^{1,3,4};¹Department of Biological Sciences, Vanderbilt University, Nashville, TN, ²Department of Mechanical Engineering, Vanderbilt University, Nashville, TN, ³Vanderbilt Institute for Integrative Biosystems Research and Education, Vanderbilt University, Nashville, TN, ⁴Department of Cancer Biology, Vanderbilt University, Nashville, TN

Non-muscle myosin II (MyoII) contractility is important to the regulation of many cellular processes, including migration. The small GTPase Rho has been shown to regulate MyoII contractility, but the role of other GTPases, such as Rac, in modulating contractility is not well understood. In this study, we show that activation of Rac by the guanine nucleotide exchange factor (GEF) Asef2 increases MyoII contractility to impair cell migration on two-dimensional (2D) type I collagen. Knockdown of endogenous Rac using short hairpin RNAs (shRNAs) or treatment of cells with the Rac-specific inhibitor NSC23766 results in a significant decrease in the amount of active MyoII, as determined by serine 19 (S19) phosphorylation, and negates the Asef2-promoted increase in contractility. Furthermore, treatment of cells with blebbistatin, which inhibits MyoII activity, abolishes the Asef2-mediated effect on migration. Because adhesion growth and maturation have been linked to actomyosin contractility, we also examined the effect of Asef2 on adhesions. Interestingly, Asef2 increases active β 1 integrin levels in adhesions and induces large, mature adhesions that assemble and disassemble (turnover) more slowly compared with controls. In addition, the Asef2-promoted increase in the level of active β 1 integrin in adhesions was diminished by treatment with the Rac inhibitor NSC23766. Because three-dimensional (3D) matrices more closely mimic the physiologic environment of cells, we investigated the role of Asef2 in regulating migration and MyoII activity in 3D environments. For these studies, we developed microfluidic devices that afford a controlled, reproducible platform for generating 3D matrices. Using these devices, we show that Asef2 inhibits cell migration in 3D type I collagen matrices, and treatment of cells with blebbistatin abolishes the Asef2-mediated decrease in migration. Moreover, Asef2 enhances MyoII activity as shown by increased S19 phosphorylation, and treatment with NSC23766 abolishes the Asef2-promoted increase in active MyoII. Collectively, these results indicate that Rac activation, promoted by Asef2, is critical for modulating MyoII activity and cell migration in both 2D and 3D environments.

P2202**Board Number: B829****Glycodelin abolishes PMA-induced migration of MCF-7 breast cancer cells.**L. Hautala¹, R. Koistinen¹, H. Koistinen¹;¹Department of Clinical Chemistry, University of Helsinki, Helsinki, Finland

Glycodelin is a secreted glycoprotein mainly expressed in well-differentiated epithelial cells in reproductive tissues. Previously, glycodelin has been shown to induce cell differentiation in endometrial and breast cancer cells. We have transfected glycodelin into MCF-7 breast cancer cells to study its effect on cell differentiation. When the cells were grown in Matrigel basement membrane preparation, glycodelin-expressing cells formed glandular structures, while the control cells were less cohesive and had less luminal structures. In addition, glycodelin-producing cells formed significantly smaller tumors in xenograft mice. The effects of glycodelin are, at least partly, mediated by repressed PKC δ activity, rendering the cells resistant to PKC δ activation by phorbol 12-myristate 13-acetate (PMA). Here we continued these previous studies by addressing the effect of glycodelin on the invasion and migration of MCF-7 breast cancer cells, and studying the affected signaling route in more detail. Activation of signaling proteins was detected with an antibody array of 43 phospho kinases. Cell migration was studied using wound healing test. The cells were grown on chamber slides until they formed a monolayer and then a scratch was made using a pipet tip. After addition of PMA or DMSO control, the migration of the cells was monitored under microscope. For control, PKC δ was down-regulated with RNAi prior to the migration assay. Boyden chamber assay was used to study the invasion through Matrigel. The cells were grown with and without PMA and the amount of invasive cells was quantified. Phospho-kinase array did not reveal any widespread changes in the amount of phosphorylated signaling molecules between the glycodelin-producing and control cells irrespective of the PMA treatment. However, in addition to previously detected decrease in PKC δ levels, increased p27 and Chk-2, and decreased p38 α were observed in glycodelin-producing cells. In wound healing test PMA caused a two-fold increase in the migration of the control cells, but had no effect on the glycodelin-producing cells. When the expression of PKC δ was down-regulated in the control cells using siRNA, the addition of PMA did not increase the migration of the cells. PMA also increased the invasiveness of the cells, but no difference was observed between glycodelin-expressing and control cells. Without PMA treatment both glycodelin-expressing and control cells behaved similarly in wound healing and invasion tests. In conclusion, glycodelin abolished PMA-induced migration in MCF-7 breast cancer cells, while it did not have any effect on the invasion of the cells. Our results suggest that the effect of glycodelin in migration is mediated by reduced activation of PKC δ .

P2203**Board Number: B830****Regulation of cancer cell motility by secreted exosomes.**B. Sung¹, T. Ketova², D. Hoshino¹, A. Zijlstra^{1,2}, A.M. Weaver^{1,2};¹Department of Cancer Biology, Vanderbilt University Medical Center, Nashville, TN, ²Department of Pathology, Microbiology and Immunology, Vanderbilt University Medical Center, Nashville, TN

Directional movement of cells through tissues is critical for embryonic development and cancer metastasis, yet how cells maintain polarity in the face of complex environmental cues is poorly understood. Here, we use intravital live imaging in the chick embryo chorioallantoic membrane (CAM) to demonstrate that secretion of exosomes from late endosomes is required for directionally persistent *in vivo* movement of cancer cells. Knockdown (KD) of three distinct exosome secretion regulators leads to defects in tumor cell migration associated with excessive formation of unstable protrusions and directional switching. Rescue experiments with purified exosomes and matrix coating identify adhesion assembly as a key function of exosomes that promotes cell motility. We propose that autocrine secretion of exosomes is a powerful way to promote directionally persistent cell motility by reinforcing otherwise transient polarization states.

P2204**Board Number: B831****Loss of Dlg5 expression promotes the migration and invasion of prostate cancer cells via Girdin phosphorylation.**L. Tomiyama¹, T. Sezaki¹, M. Matsuo¹, K. Ueda^{1,2}, N. Kioka¹;¹Agriculture, Kyoto University, Kyoto, Japan, ²iCeMS, Kyoto University, Kyoto, Japan

Dlg5 has been reported to participate in cancer progression; however, its role in prostate cancer still remains poorly understood. In this study, we demonstrate that Dlg5 is frequently downregulated in prostate cancer. We show here that Dlg5 is involved in the regulation of cell migration and cancer cell invasion. Knockdown of endogenous Dlg5 markedly increased prostate cancer cell migration and invasion. Our studies, for the first time, demonstrate the interaction between Dlg5 and Girdin, an actin-binding Akt substrate. Importantly, we found that levels of Akt-mediated Girdin phosphorylation (p-Girdin-Ser1416) are increased in Dlg5 -depleted cells. Small interfering RNA directed against Girdin and wortmannin treatment, which was found to reduce Girdin phosphorylation, impaired the effect of Dlg5 depletion on cell migration. Taken together, our findings demonstrate that Dlg5 interacts with and inhibits the activity of Girdin, thereby suppressing the migration of prostate cancer cells.

P2205**Board Number: B832****Novel biosensors for Dbl family RhoGEFs elucidate control of Rac1 by multiple GEFs during breast cancer cell migration.**D.J. Marston¹, M. Vilela², G. Danuser², J. Sondek¹, K.M. Hahn¹;¹Department of Pharmacology, University of North Carolina, Chapel Hill, NC, ²Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX

Dbl-family RhoGEFs are broadly relevant signaling molecules that regulate cytoskeletal dynamics by integrating the effects of growth factors and adhesion molecules. The timing and localization of GEF activity is precisely controlled to generate different Rho family GTPase responses, thereby producing different actin dynamics and specific types of cellular protrusions. We describe a novel design approach, capable of generating biosensors for multiple GEFs, applied here to visualize activation of Vav2, ASEF and Tiam1 in living cells. A pair of fluorescent proteins was inserted in a region of the GEF with no known binding activity, such that relief of GEF autoinhibition altered FRET between the fluorescent pair. High content screening assays were used to optimize donor/acceptor pairs, insertion site, linkers and circular permutants, increasing the dynamic range of the biosensors to produce FRET ratio changes of up to 300%. The Vav2 biosensor reported phosphorylation of Vav2 by Src kinase and reversible Vav2 activation upon EGF stimulation. This biosensor was used to examine activation dynamics during constitutive migration of MDA-MB-231 breast cancer cells, revealing periodic activation of Vav2 during protrusion retraction cycles at the leading edge. Current studies using computational multiplexing are examining the coordination of Vav2 and Tiam1 to regulate Rac-mediated protrusion and cell polarization.

P2206**Board Number: B833****“A Novel Approach for Identifying Cytohesin Variants and their Isoforms in HGF Stimulated Cell Migration using High Resolution Melting Analysis.”.**A. Taylor¹, L.C. Santy¹;¹Department of Biochemistry, Microbiology and Biochemistry, Pennsylvania State University, University Park, PA

Cytohesins are guanine nucleotide exchange factors that play major roles in cell migration, adhesion, and cytoskeleton recycling. The cytohesin family members include: cytohesin1, cytohesin2/ARNO, cytohesin3/Grp1, and cytohesin4. They function by controlling the activation of small GTPases ADP ribosylation factors (ARF1 through 6). These members are closely related and share a similar domain structure. The NH2 terminal domain is comprised of a ~60 residue coil-coil domain that mediates homodimerization scaffolding and localization properties. The central ~200 amino acid catalytic domain is immediately followed by a PH domain. The pleckstrin homology (PH) domain of each of these proteins

is the key determinant of the phosphoinositide binding affinity of these PH domain. Cytohesins undergo alternative splicing of a single three- nucleotide exon, resulting in isoforms containing either a diglycine or triglycine motif in their PH domain. We have previously demonstrated that the number of glycine residues in the PH domain of cytohesin2/ARNO and cytohesin3/Grp1 determines their effects on cell adhesion, spreading and localization. It is important to be able to distinguish between the diglycine or triglycine variants in order to help elucidate their roles in cells. To date, an effective assay to differentiate the diglycine or triglycine splice variants has not been developed. High Resolution Melting (HRM) analysis has recently become an efficient, quick, and robust method for genotyping, mutation scanning, and sequencing matching. HRM has proven to be very efficient in even identifying single nucleotide polymorphisms. This study investigated whether HRM analysis can be used to identify and distinguish between cytohesin2/ARNO and cytohesin3/Grp1 and their respective "2G" and "3G" isoforms in madin darbin canine kidney epithelial cells. Specifically, we employed HRM amplicon melting, HRM "snapback" genotyping (asymmetric and symmetric), and unlabeled probe genotyping analyses. The cytohesin amplicons were cloned and sequenced to assist with the interpretation of HRM data. The cDNA HRM curves correlate well with the DNA plasmid controls, which either contained Cytohesin2/ARNO "2G" or "3G" and Cytohesin3/Grp1 "2G" or "3G". We show that HRM coupled with LC-Green-PCR is a robust, closed-tube, inexpensive analysis for the rapid detection of canine cytohesin2/ARNO and cytohesin3/Grp1 and their respective isoforms. By combining HRM with a simple PCR design, this novel approach is the first assay capable of distinguishing between cytohesin splice variants. Cytohesins are important regulators of proliferation, migration and invasiveness in cancer. This study provides an assay to identify the specific isoforms that are being upregulated, and thus aid in the understanding of cytohesin function in cancer. In addition, identifying cytohesin isoforms as prospective therapy targets for different cancer types and other diseases that affect cell motility could be possible.

P2207

Board Number: B834

Haptotaxis requires a Rac1/WAVE/Arp2/3-based pathway that regulates differential lamellipodial dynamics.

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Cells in vivo migrate in response to directional cues such as differing concentrations of extracellular matrix (haptotaxis); however, a mechanistic understanding of how cells sense and respond to these haptotactic cues is lacking. In order to generate the forces required to drive cell migration, actin filaments must be nucleated and organized in a precise manner. One primary mechanism by which new actin filaments are nucleated is the Arp2/3 complex. Using a small molecule inhibitor of the Arp2/3 complex, CK666, and Arp2/3-depleted cells we have previously shown that the Arp2/3 complex is essential for haptotaxis in fibroblasts on a variety of extracellular matrices (Wu, C., S. B. Asokan et al,

Cell, 2012). In order to dissect the mechanism of haptotaxis on fibronectin, an agnostic approach using microfluidic chambers that allow direct imaging of fibroblast haptotaxis on gradients of fibronectin has been utilized. The requirement for the Arp2/3 complex for haptotaxis on fibronectin was confirmed using CK666 on vascular smooth muscle cells, indicating that this result is not cell type specific. It has been proposed that the alignment and maturation of focal adhesions could regulate haptotaxis. However, our experiments suggest that fibronectin based haptotaxis of fibroblasts does not appear to be reliant upon a specific integrin pairing or upon the presence of 'mature' focal adhesions. One important nucleation promoting factor (NPF) for Arp2/3 is the WAVE complex. Our data suggests that the WAVE complex is the primary NPF required for fibronectin based haptotaxis, with N-WASP and WASH being dispensable. Rac1 is required for WAVE activation, lamellipodial formation and is a component of focal complexes. Inhibition or depletion of Rac1 abrogates fibronectin haptotaxis. Additionally, inhibition of other focal complex components, Src or FAK, also blocks fibroblast haptotaxis on fibronectin, implicating focal complexes in the regulation of fibronectin haptotaxis. Kymography has revealed differential protrusion dynamics across haptotaxing cells, which have the potential to provide the force necessary for differential migration. Furthermore, FRAP studies investigating actin dynamics during haptotaxis have revealed that there are differential actin dynamics across a cell on a gradient on fibronectin that is not present in a cell on uniform fibronectin or upon Arp2/3 inhibition. Our current hypothesis is that differential engagement of integrins triggers a Src/Rac/WAVE pathway that leads to Arp2/3 activation. Resulting in differential actin dynamics and protrusion dynamics, ultimately leading to directed migration towards higher concentrations of extracellular matrices.

P2208

Board Number: B835

A simple assay that distinguishes cell migration from proliferation.

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Cell motility is a fundamental characteristic of most mammalian cell types and plays a crucial role in normal and pathological processes such as development, wound healing, immune response, and cancer metastasis. Migration is therefore one of the most commonly assayed cellular behaviors. There are a variety of techniques used to evaluate this phenomenon, which range in cost, reliability, and necessary technical expertise. One of the most popular methods is the barrier, or void-filling, assay in which cells are seeded on a surface within a restricted area then allowed to migrate into an adjacent space. This approach is inexpensive, relatively high throughput, can be accomplished with widely available equipment, and does not require advanced expertise or training. The readout is the number of cells that are located in the initially cell-free zone at the end of the migration period. One significant drawback of this approach is that the readout necessarily represents the combined effects of both cell migration and proliferation. We have developed a simple alteration to this approach that employs a commercially available, fluorescent, live-cell stain that is retained through cellular generations. Cell motility is

measured as the ratio of the fluorescent signal in the migration area relative to the seeding and migration areas combined. This method allows the contribution of cell migration to be directly measured without the confounding effects of proliferation. We demonstrate the efficacy of this method with cells that have both high and low proliferation or migration rates. We also show that this method can be used to directly compare migration rates between different cell types.

P2209

Board Number: B836

Arpin-mediated idling and reductions in cell speed are associated with turns.

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We recently reported a novel protein, Arpin, which inhibits the Arp2/3 complex, a molecular machine that nucleates actin filaments at the cellular front in the lamellipodium. There, new actin filaments polymerize and exert force to protrude the edge forward. Arp2/3-based positive feedback signaling is the basis for lamellipodial persistence, which drives effective directional migration. Indeed, we observed that Arpin is associated with decreases in two major parameters of cell migration: speed and directional persistence. To understand how Arpin controls these parameters, in this work we re-examined migration trajectories of three different cell types with Arpin perturbations. In all three systems, we found that speed and turn angle are negatively correlated, suggesting a universal linkage between these parameters. Arpin-mediated decreases in instantaneous speed were associated with increases in turn angle. Importantly, in all three systems Arpin induced episodes of idling, defined as moving below a speed threshold. This threshold is a percentage of the average speed, calculated using the first derivative of log mean squared displacements. Observed idling episodes interrupted and effectively shortened duration of the active phase of migration, consistent with a model in which Arpin counteracts a positive-feedback circuit that sustains the lamellipodium over time. Finally, Arpin-associated idling episodes were followed by turns, implicating idling as a means for enacting a turn.

P2210

Board Number: B837

The Role of Cytohesins in Cell Migration After Acute Kidney Injury.

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Acute kidney injury (AKI) is a common and potentially fatal complication that affects 5-9% of hospitalized patients, and with a greater incidence (> 10%) in intensive care unit patients every year. The molecular aspects of kidney injury and repair are still uncertain. Studies have shown that hepatocyte growth factor (HGF) promotes recovery of the injured kidney by inducing survival and migration of

tubular epithelial cells to repopulate the bare tubule areas. We have demonstrated *in-vitro* that HGF-stimulated kidney epithelial cell migration requires the activation of Arf6 via the cytohesin family of Arf guanine nucleotide exchange factors (GEFs), and the subsequent activation of Rac1. We hypothesize that this signaling module is required to promote the HGF-stimulated recovery of damaged kidney. We have established an ischemia and reperfusion injury (IRI) mouse model to analyze the effects of modulating this signaling pathway on kidney recovery. We have observed that treatment of injured mice with HGF results in lower creatinine (Cre) and blood urea nitrogen (BUN) when compared to untreated IRI mice, indicative of a faster kidney recovery. In addition, histological analysis of injured kidney tissue samples show that HGF treatment promotes recovery by enhancing kidney tubule repair. We have also treated IRI mice with the cytohesin inhibitor SecinH3. Cre and Bun levels as well as histological analyses show that, unlike HGF, SecinH3 treatment halts injured kidney recovery and delays repair when compared to untreated IRI mice. Analyses to detect active Rac1 and active Arf6 are being carried out to further determine the role of cytohesins and Arf6 in HGF-stimulated kidney recovery. Nevertheless, our results suggest a role of cytohesins in renal ischemia and reperfusion injury repair. We believe that HGF-stimulated cell migration in the injured kidney requires the activation of cytohesin. By testing our hypothesis, we expect to broaden the understanding of cell migration in AKI recovery.

P2211

Board Number: B838

The Role of Active ARF6 in Cytohesin-Induced Rac Activation.

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Hepatocyte growth factor (HGF) is a potent signaling molecule that causes epithelial cells to scatter and migrate. Small GTPases play an important role regulating the changes downstream of HGF that lead to the initiation of migration. For example, crosstalk between the small GTPases ARF6 and Rac stimulates epithelial cell migration. The ARF-GEF Cytohesin-2 and the Rac-GEF Dock180 have also been implicated in HGF signaling as treatment with the cytohesin inhibitor SecinH3 or expression of the dominant negative form of Dock180 impairs HGF-stimulated wound healing. We have previously shown that the GEF activity of Cytohesin-2 and its promotion of a complex containing the scaffolding protein GRASP and Dock180 is required for cytohesin-induced Rac activation. However, the role of active ARF6 in this process is unclear. Using live-cell imaging, we found that GRASP and Dock180 co-localize with the recycling endosome markers EHD1 and Rab11 at internal locations. Here, we show that upon stimulation, exogenously expressed GRASP and Dock180 translocate from their internal location to the cell periphery when Cytohesin-2 is also expressed. In addition, we are able to detect a FRET signal in live cells using GRASP and Dock180 as FRET pairs, indicating that we can now use FRET to study the spatial and temporal dynamics of the complex. We hypothesize that activation of ARF6 by Cytohesin-2 regulates trafficking of GRASP and Dock180 from recycling endosomes to the plasma membrane. As either overexpression or up-regulation of HGF has been implicated in both cancer progression and

wound healing, respectively, increased understanding of HGF signaling would be beneficial to lead to the development of novel therapeutics to either fight metastasis or promote injury recovery.

P2212

Board Number: B839

A NOVEL MICROFLUIDIC ASSAY THAT MIMICS THE IN VIVO LEUKOCYTE-ENDOTHELIUM INTERACTIONS.

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Due to its significance, several in vitro models have been developed to study different aspects of the leukocyte adhesion cascade. However, currently none of these in vitro models can be used for real-time studies of the entire leukocyte adhesion cascade including rolling, adhesion and migration in a single assay. In this study, we have developed and validated a novel bioinspired microfluidic assay (bMFA) and used it to test the hypothesis that blocking of specific steps in the adhesion/migration cascade significantly affects other steps of the cascade. The bMFA consists of an endothelialized microvascular network in communication with a tissue compartment via a 3 μm porous barrier. Human neutrophils in bMFA preferentially adhered to activated human endothelial cells near bifurcations with rolling and adhesion patterns in close agreement with in vivo observations. When the tissue compartment of bMFA was filled with fMLP, 50% of adhering neutrophils migrated into the tissue compartment after 120 min. Treating endothelial cells with monoclonal antibodies to E-selectin or ICAM-1 or treating neutrophils with wortmannin reduced rolling of neutrophils to $\sim 60\%$, adhesion to $\sim 20\%$ and migration to $\sim 18\%$ of their respective control values. Furthermore, antibody blocking of specific steps in the adhesion/migration cascade (e.g. mAb to E-selectin) significantly downregulated other steps of the cascade (e.g. migration). This novel in vitro system provides a realistic human cell based model for basic science studies, identification of new treatment targets, selection of pathways to target validation, and low-cost test bed for rapid screening of candidate agents.

Dynamics of Focal Adhesions and Invadosomes

P2213

Board Number: B840

Linking sub-cellular mechanical forces to integrins mobility using Image Correlation Spectroscopy.

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The ability of cells to sense physical properties of the extracellular matrix (ECM) is essential to regulate their proliferation, migration and differentiation. Cells feel the different properties of ECM and respond to it by exerting forces on their microenvironment. This process is mainly based on the activity of integrin receptors. Integrin activities depend on their ability to bind-unbind cyclically ECM and are then characterized by their diffusion regime at the plasma membrane (Rossier & al, 2012) which might be modulated by active forces.

The present project aims to understand the link between cellular forces and integrin activity in the regulation of mechanotransduction processes. Our work focuses on 2 types of integrins: b_1 that plays a role as force generator and b_3 that rather regulates cellular forces (Roca-Cusachs & al, 2009). The strategy adopted consists in modulating the spatial distribution of cellular forces by using micropatterns of various geometries and coupling forces modulation to temporal Image Correlation Spectroscopy (tICS). Practically, tICS allows us to determine in any point of the cells the mobility and concentration of b_1 and b_3 integrins tagged with GFP. The principle of tICS is based on the temporal autocorrelation of a series of images. From the amplitude and the shape of the autocorrelation functions inside a given ROI, we can extract information about the molecule number, their mean diffusion constant and the immobile molecule fraction. Each chosen ROI corresponds to a particular force arrangement, previously measured using patterned traction cytometry (Tseng & al, 2011).

Based on our set-up, we were able to modulate the spatial distribution of actin generated forces at the single cell level. We then measured the relation between those sub-cellular forces and the local concentration and mobility of those integrins. Interestingly, we found a strong correlation between the amplitude of traction forces and the mobility of b_3 , confirming its role of regulator in cell mechanical activity. Strikingly, we did not find the same behaviour in the case of b_1 integrins. Overall, this work brings new insights in the field of cell mechanics and provides information at the cell level about integrin dynamics and their correlation to cell mechanical activity.

P2214**Board Number: B841****Structured illumination microscopy reveals that focal adhesions are composed of linear subunits.**S. Hu¹, Y. Tee¹, A. Kabla², R. Zaidel-Bar¹, A.D. Bershadsky^{3,4}, P. Hersen^{1,5};¹Mechanobiology Institute, Singapore, Singapore, ²Department of Engineering, University of Cambridge, Cambridge, United Kingdom, ³Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel, ⁴Mechanobiology Institute, National University of Singapore, Singapore, Singapore, ⁵Université Paris Diderot, Laboratoire Matière et Systèmes Complexes, UMR 7057 CNRS, Paris, France

The cells ability to mechanically interact with the extracellular matrix (ECM) is a fundamental feature of adherent eukaryotic cells, and is required for cell migration, proliferation, and differentiation. Adhesion is mediated by protein complexes, called focal adhesions. Recent progress in super resolution microscopy revealed that they have an internal organization, yet such methods do not allow observing the formation and dynamics of focal adhesions' internal structure. Here, we combine structured illumination microscopy (SIM) with total internal reflection fluorescence microscopy (TIRF) to be able to observe focal adhesions at high spatial resolution in live cells. We show that focal adhesion proteins inside focal patches are distributed along elongated subunits, typically 300 nm wide, separated by 300 nm and individually connected to actin cables. These focal adhesions subunits growth speed is in average of 0.3 $\mu\text{m}/\text{min}$. We further show that these structures are intimately linked to actin radial fiber formation. Taken together, our study reveals that, when observed with an appropriate resolution in both space and time, the minimal units of adhesion appear to be elongated, dynamic structures. We anticipate this ultrastructure to be relevant when studying the mechano-biological attributes of focal adhesions and their behavior in response to mechanical stress.

P2215**Board Number: B842****Development of a novel cellular gel substrate system and imaging platform for super-resolution fluorescence microscopy.**V.K. Gupta^{1,2}, M.J. Colville^{3,4}, A. Singh⁵, W.R. Zipfel^{3,5,6}, M.J. Paszek^{2,3,7};¹Sibley School of Mechanical and Aerospace Engineering, Cornell University, Ithaca, NY, ²School of Chemical and Biomolecular Engineering, Cornell University, Ithaca, NY, ³Field of Biophysics, Cornell University, Ithaca, NY, ⁴Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY, ⁵School of Applied and Engineering Physics, Cornell University, Ithaca, NY, ⁶Department of Biomedical Engineering, Cornell University, Ithaca, NY, ⁷Kavli Institute at Cornell for Nanoscale Science, Cornell University, Ithaca, NY

Cells sense the stiffness of their external environment and use this information to influence cell differentiation, tissue development, and pathological states, including cancer. Large protein assemblies

called focal adhesions are primarily responsible for linking the cell to the extracellular matrix (ECM) and converting external mechanical forces into internal biochemical signals. Although recent developments in super-resolution fluorescence microscopy allow one to study the nanoscale architecture of focal adhesions, these studies have only been conducted on glass, due to the challenge of nanometer imaging through standard gel substrates. Our aim was to develop a novel imaging platform and high refractive index gel substrate system of tunable stiffness for super-resolution imaging in both the lateral and vertical dimensions. The gel system that we developed consists of a commercially available silicon elastomer whose stiffness can be systematically varied by altering the ratio of its two components. The fabrication of the gels was optimized to achieve uniform thickness, an even surface, high optical quality and transparency, and minimal background fluorescence, which are critical for super-resolution imaging. The elastic moduli of the gels were measured using Dynamic Mechanical Analysis. By varying the ratio of the two gel parts, we were able to systematically adjust the elastic moduli of our gels from 1.7 kPa to 21 kPa. The gel system was highly compatible with cell culture. We observed that epithelial cells demonstrated similar spreading and growth characteristics on stiff (~21 kPa), fibronectin-conjugated gels compared to glass substrates conjugated similarly.

We next developed a custom imaging platform for Photo-activated localization Microscopy (PALM) imaging of adhesion structures in cells on our gel substrates. Our system employed a custom circular scanning Total Internal Reflection Fluorescence Microscopy (TIRFM) system, in which the focus of the laser beam is scanned around the periphery of the focal plane, to compensate for irregularities of the gel surface and unevenness of the TIRF field. By combining our imaging platform and elastomer system, we achieved high signal-to-noise imaging of individual fluorophores in PALM. As a proof of principle, super-resolution images of paxillin-mEos2 in epithelial cells were acquired on our elastomeric substrates. We now are evaluating quantitatively how substrate stiffness impacts the molecular organization of major focal adhesion proteins including FAK, vinculin, paxillin, and talin. This work will lead to a greater understanding of how adhesion complexes sense and respond to the stiffness of their environment.

P2216

Board Number: B843

Kinesin Motor Kid Plays a Role in Cell Adhesion and Migration by Affecting Focal Adhesion Localization and Dynamics.

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The kinesin-10 chromokinesin motor family is traditionally considered to play a role mainly during mitosis influencing chromosome alignment. We believe the Kid/KIF22 member of this group has additional functions during interphase of the cell cycle.

We observed that Kid localized to the sites of focal adhesions (FAs) in multiple cell lines and FAs became redistributed when Kid's function was impaired. In wild-type HeLa cells, FAs are mostly distributed near the peripheral edges of the cell. After knockdown of Kid, we found that the number of FAs significantly increased, and they became more scattered throughout the cytoplasm. FAs are integrin-based multiprotein structures that mediate the attachment of cells to the extracellular matrix (ECM). They play essential roles in various cellular processes including cell morphology, migration, proliferation, and differentiation. Hence, we checked if such changes in the number and distribution of FAs have any effect on cell adhesion and/or migration. Indeed, cells after knockdown of Kid showed a much slower rate of spreading compared to control cells when seeded onto fibronectin, which is a component of ECM. Additional results from our wound healing assay suggested that loss of Kid caused an about two-thirds reduction in the rate of cell migration.

Based on these observations, and previous studies showing the role of microtubules in FA stability, we hypothesized that Kid might be involved in regulating assembly and/or disassembly of FAs, thus affecting cell adhesion and migration. Confocal microscopy showed a higher proportion of focal complexes (FCs), which are smaller precursors of FAs, after knockdown of Kid. TIRF microscopy indicated that loss of Kid led to an increased life duration time of FAs. Since the small GTPase Rho is required for the maturation of FCs to FAs, we tested if the regulation of FA dynamics by Kid is Rho-dependent. When wild-type HeLa cells are treated with Rho inhibitor, there were few FAs due to the inhibition of FA maturation. Moreover, there were few FCs as the Rho inhibitor also interrupts actin stress fibers which are required for the assembly of FCs. Interestingly, when we treated cells with Rho inhibitor after knockdown of Kid, there were more FAs and FCs remaining, supporting the idea that Kid functions to destabilize FAs in the absence of Rho activity.

Taken together, these data suggested that Kid plays a role in the localization and dynamics of adhesion structures. This study identified a non-conventional role of Kid during interphase and expanded on our knowledge of how a motor protein might be involved in the focal adhesion network affecting cell adhesion and migration.

P2217

Board Number: B844

Novel function of kinesin KIF1C as a component of podosome adhesive rings in vascular smooth muscle cells.

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Extracellular matrix remodeling during physiological processes is mediated by invasive protrusions called podosomes. Podosomes consist of a polymerizing actin core surrounded by an adhesive ring. In vascular smooth muscle cells (VSMCs), podosomes can be rapidly initiated by PKC activation. We have previously

shown that kinesin KIF1C is important for efficient podosome formation in VSMCs but the mechanism whereby KIF1C performs this function is largely unknown.

Now, our live-cell imaging data show that deposition of KIF1C at distinct sites at the ventral cell membrane correlates with new podosome formation at those sites. In particular, KIF1C accumulates around emerging podosome cores, which are marked by cortactin and TKS5, and acquires a ring-like shape. Our data indicate that KIF1C is delivered and relocated along MTs, but resides at adhesive rings in a MT-independent manner. Because without KIF1C, both the number and size of podosomes are decreased, we hypothesize that this kinesin provides a base for the adhesive ring formation. In addition, KIF1C likely supports relocation of adhesive ring components during movement of existing podosomes to new cellular positions, as seen in relocation of podosomes from the periphery toward the cell center via KIF1C-rich membrane tubes.

These findings indicate that microtubule-dependent KIF1C transport and deposition is critical for podosome positioning in VSMCs. Through this mechanism, our study implicates KIF1C as a regulator in the distribution of podosomes over the ventral cell surface that is thought to be important for a more uniform ECM remodeling by VSMCs rather than site specific degradation typical for cancer cells.

P2218

Board Number: B845

Microtubules Regulate Focal Adhesion Dynamics through MAP4K4.

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Focal adhesions are dynamic organelles that establish a connection between the ECM (extracellular matrix) and cytoskeletal networks and serve as points of traction for cells. The disassembly of focal adhesions allows cell retraction and integrin detachment from the ECM, processes critical for cell movement. A long-standing and intriguing observation is that growth of MT filaments in migratory cells can be guided toward focal adhesions, and targeting of MT growing ends (plus ends) to focal adhesion often precedes focal adhesion turnover. It has been speculated that MTs can serve as tracks to deliver proteins essential for focal adhesion disassembly. However, the molecular nature of the focal adhesion “disassembly factor” remains elusive. By quantitative proteomics, we identified mammalian MAP4K4 (mitogen-activated protein kinase kinase kinase kinase 4) as a focal adhesion regulator that associates with MTs. Conditional knockout (cKO) of MAP4K4 in skin epidermal cells stabilizes focal adhesions and impairs epidermal migration. Exploring underlying mechanisms, we further show that MAP4K4 associates with MT plus end tracking protein, EB2 (end binding 2) and an Arf6-specific guanine nucleotide exchange factor, ARF-GEP100 (ADP-Ribosylation Factor-Guanine Nucleotide Exchange Protein-100 kDa), which is also known as IQSEC1 (IQ motif and SEC7 domain-containing protein 1). Together, our findings provide new insights for this critical cellular process, suggesting that EB2-containing MT plus ends can deliver MAP4K4 toward focal adhesions, where MAP4K4 can in turn activate Arf6 via IQSEC1 and enhance focal adhesion dissolution.

P2219**Board Number: B846****Phosphorylation of Serine 106 Regulates Asef2 GEF Activity and Asef2-Mediated Cell Migration and Adhesion Turnover.**B. Erdogan¹, J. Evans¹, K. Hines^{2,3,4}, J. Forsythe^{2,3,4}, M. Shi¹, S. Hill⁵, K. Rose^{5,6}, J. McLean^{2,3,4}, D. Webb^{1,4,7};¹Department of Biological Sciences, Vanderbilt University, Nashville, TN, ²Department of Chemistry, Vanderbilt University, Nashville, TN, ³Vanderbilt Institute for Chemical Biology, Vanderbilt University, Nashville, TN, ⁴Vanderbilt Institute for Integrative Biosystems Research and Education, Vanderbilt University, Nashville, TN, ⁵Mass Spectrometry Research Center, Vanderbilt University, Nashville, TN, ⁶Department of Biochemistry, Vanderbilt University, Nashville, TN, ⁷Department of Cancer Biology, Vanderbilt University, Nashville, TN

Asef2 is a recently identified guanine nucleotide exchange factor (GEF) that regulates cell migration and adhesion turnover via activation of Rho family GTPases, including Rac. Asef2 is activated by binding of the tumor suppressor adenomatous polyposis coli (APC) to its N-terminal region; however, much less is known about other modes of Asef2 regulation, such as post-translational modifications. In this study, we identified six phosphorylation sites in Asef2 using liquid chromatography coupled with high-resolution mass spectrometry (LC-MS) and tandem mass spectrometry (MS/MS). One of these phosphorylation sites, serine 106 (S106), is located within the APC binding region, suggesting a potential role in Asef2 regulation. To probe the effects of S106 phosphorylation on Asef2 activity, Asef2 mutants were generated in which S106 was changed to either alanine (S106A) or aspartic acid (S106D), which represent non-phosphorylatable and phosphomimetic analogues, respectively. GFP-tagged Asef2 S106 mutants were transfected into HT1080 cells and activation of Rac was assessed using a pulldown assay. S106A mutation greatly reduced Rac activation by Asef2, while the phosphomimetic mutation (S106D) led to an increase in active Rac levels. Next, we tested the effects of S106 mutation on cell migration using live-cell time-lapse microscopy. Wild-type GFP-Asef2 promoted migration of HT1080 cells on fibronectin, as we have previously shown. Expression of GFP-Asef2-S106A abrogated Asef2-promoted migration, whereas GFP-Asef2-S106D expression promoted migration similarly to GFP-Asef2. Because our previous study showed that Asef2 promotes cell migration by increasing adhesion assembly and disassembly (adhesion turnover), we next determined if S106 phosphorylation affects cell migration by modulating adhesion turnover. Cells expressing wild-type GFP-Asef2 showed an approximately 50% decrease in the $t_{1/2}$ values for adhesion assembly and disassembly compared to control cells expressing GFP, indicating that adhesions turn over more rapidly in Asef2-expressing cells. Conversely, expression of GFP-Asef2-S106A led to an increase in the $t_{1/2}$ values for adhesion assembly and disassembly compared to GFP-Asef2 expression, indicating that S106A expression negates the Asef2-mediated effect on adhesion turnover. Moreover, expression of GFP-Asef2-S106D caused more rapid adhesion turnover comparable to that observed with GFP-Asef2 expression. These results demonstrate that Asef2 S106 phosphorylation promotes faster adhesion turnover, which is critical for efficient cell migration. Taken together, we have identified a new regulatory mechanism for Asef2, S106 phosphorylation, which is crucial for Asef2-mediated Rac activation, cell migration, and adhesion turnover.

P2220**Board Number: B847****Septins enhance the mesenchymal-like motility of renal epithelia by promoting stress fiber connectivity and focal adhesion maturation.**L. Dolat¹, J.L. Hunyara¹, J.R. Bowen¹, E.P. Karasmanis¹, V. Galkin², E.T. Spiliotis¹;¹Biology, Drexel University, Philadelphia, PA, ²Physiological Sciences, Eastern Virginia Medical School, Norfolk, VA

Epithelial-mesenchymal transition (EMT) underlies renal fibrosis and the metastasis of renal cell carcinomas (RCCs). EMT is characterized by disruption of cell-cell adhesions, cell scattering and the enhancement of cell motility with a front-rear polarity. While the disruption of cell-cell adhesion during EMT is extensively studied, little is known about the molecules and mechanisms that enhance the mesenchymal-like motility of epithelial cells. Septins are filamentous G proteins that are over-expressed in metastatic RCCs, but their functions in the mesenchymal-like motility of epithelial cells is unknown. Using the Madin-Darby canine kidney (MDCK) model of partial EMT that allows the study of epithelial motility in 2D and 3D matrices after stimulation with hepatocyte growth factor (HGF), we discovered a novel network of septin filaments that underlies the organization of the transverse arc and radial (dorsal) stress fibers in the leading lamella of migrating epithelia. We show that this lamellar network of septin filaments is required for the maturation of nascent focal adhesions as septin depletion results in smaller and more transient and peripheral focal adhesions with increased levels of phosphorylated paxillin-Y118. In addition, septin-depleted cells consisted of a disorganized transverse arc and shorter and fewer radial stress fibers. These phenotypes were independently rescued by alpha-actinin and the actin-binding motor-dead myosin II N93K, but not by alpha-actinin-deltaABD, which cannot bind or bundle actin, or the constitutively active myosin regulatory light chain RLC-DD. These data suggested that septins function as actin-binding and cross-linking proteins. Using low speed sedimentation assays and negative stain EM, we show that pre-assembled actin filaments are bundled by septin 9 (SEPT9), whose expression is increased after induction of renal epithelial motility with the hepatocyte growth factor. We show that SEPT9 over-expression enhances renal cell migration in 2D and 3D matrices, while SEPT9 knock-down decreases the velocity and alters the mesenchymal-like shape of epithelial cells migrating in 3D matrices. Taken together, these results suggest that septins promote epithelial motility by reinforcing the connectivity of lamellar stress fibers and thereby, stabilizing nascent focal adhesions in the leading edge of motile epithelia.

P2221**Board Number: B848****Evidence of multiple roles for Diaphanous-Related Formins (DRFs) in Invasion.**E.W. Miller¹, S.D. Blystone¹;¹Department of Cell and Developmental Biology, SUNY Upstate Medical University, Syracuse, NY

In cancer cells, the actin cytoskeleton performs multiple functions in invasion and metastasis, partially through F-actin dense, matrix-degradation structures known as invadopodia. Assembly of monomeric actin into filaments is regulated by several different proteins, including a relatively new group of proteins known as formins. A subfamily of this group of proteins, the diaphanous-related formins (DRFs), share a common domain architecture and interact directly with Rho-family GTPases. Using quantitative real-time reverse transcriptase PCR (qRT-PCR), we determined expression levels for all ten DRFs in the highly-invasive breast cancer cell line MDA-MB-231. We then questioned whether depletion of endogenous DRF expression would result in impaired MDA-MB-231 cell invasion. Using DRF-targeting siRNA, we silenced expression of endogenous Dia1, Dia2, and FMNL1 by 75.2%, 65.0%, and 83.5% respectively. Cells treated with DRF-targeting siRNA were then subjected to an *in vitro* Matrigel invasion assay. Interestingly, knockdown of Dia1 and FMNL1 significantly inhibited invasion while knockdown of Dia2 exhibited only moderate inhibition. This led us to speculate that DRFs may have different roles in the invasion process. Using a combination of two DRF-targeting siRNA oligonucleotides, we simultaneously knocked down three combinations of DRFs, all of which had a significant, but neither all additive nor all compensative, effect on the invasive capacity of MDA-MB-231 cells. In order to determine DRF function within the cell, we used immunofluorescence-based microscopy to understand sites of activity. Interestingly, we found both endogenous FMNL1 and Dia2 to be dispersed throughout the cytoplasm with some localization to the perinuclear region of the cell. We then proceeded to investigate one of these DRFs, FMNL1, more closely. Since knockdown of FMNL1 resulted in a significant inhibition of 3D invasion, we questioned whether knockdown would have the same effect on 2D migration. Cells were treated with DRF-targeting siRNA and subjected to a scratch-wound assay. While cells treated with control siRNA closed the wound within 18 hours, cells treated with FMNL1-targeting siRNA required >24 hours to complete wound closure. FMNL1 is of particular interest as three alternative splice isoforms of this protein have been identified, all of which we detected in MDA-MB-231 cells using PCR. Future examination of all ten DRFs, especially FMNL1, and subsequent meta-analysis will allow us to determine whether there are unique events specific to individual formins which may be targeted by novel therapeutics to treat metastasis.

P2222**Board Number: B849****A plastic relationship between vinculin-transmitted tension and adhesion area defines adhesion complex size and lifetime.**U. Berge¹, P. Hernandez Varas², J.G. Lock¹, S. Strömblad²;¹Dept. of Biosciences and Nutrition, Karolinska Institutet, Huddinge, Sweden, ²Department of Biosciences and Nutrition, Karolinska Institute, Stockholm, Sweden

Cell-matrix adhesions mediate critical mechanical force transduction, but the interplay between force and adhesion regulation remains unclear. Here, live cell imaging enabled the mapping of time-dependent cross-correlations between mechanical tension and adhesion area, revealing a high degree of plasticity in this relationship. Interestingly, while an expected positive cross-correlation dominated in mid-sized adhesions, small and large adhesions displayed negative cross-correlation. Furthermore, while large changes in adhesion area followed tension alterations, small increases in area preceded tension dynamics. Modelling based on this mapping of the tension-adhesion area relationship confirmed its biological validity, and indicated that the tension-adhesion area relationship can explain adhesion size and lifetime limits, keeping adhesions focal and transient. We also identified a subpopulation of adhesions that remain stable in size and tension, whose disassembly may instead be microtubule-mediated. In conclusion, we define a plastic relationship between tension and adhesion area that controls cell-matrix adhesion focality.

Integrins and Cell-ECM Interactions 2**P2404****Board Number: B851****Nitric oxide plays a role in transmitting biochemical signal from the extracellular matrix for mammary epithelial morphogenesis.**S. Furuta¹, M.J. Bissell¹;¹Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA

Nitric oxide (NO) is a short-lived, gaseous signaling molecule produced in response to biochemical or mechanical stress in diverse tissues and organisms. Despite its evolutionary conservation and ubiquitous expression, the bioactivities of NO have been primarily studied in specialized tissues, including mammalian neurons, muscles, endothelial or immune cells, where NO triggers cell membrane hyperpolarization and relaxation or exerts cytotoxic action. In the past decade, however, different functions of NO in other tissues and organisms have been gradually unraveled, including embryogenesis and morphogenesis of *Xenopus*, pond snail and *Drosophila*. Nevertheless, whether NO is involved in the establishment of a mammalian tissue is yet to be determined. We found that non-malignant mammary epithelial cells (MECs) produced NO in response to the extracellular matrix (ECM) proteins laminin-5 and -1 and that NO production was essential for their ability to form growth-arrested polarized acini (the functional unit of mammary gland) in three-dimensional (3D) laminin-rich (1r) ECM cultures. While this mechanism was abrogated in breast cancer cells that failed to form acini in 3D cultures, induced NO production by the use of an NO donor allowed them to restore the formation of normal-like polarized

colonies. We further found that such an essential role of NO in mammary acinar formation was attributed to its ability to trigger cell membrane hyperpolarization and hence enhance tight junctions through co-localization of E-cadherin and cortical actin. These observations, for the first time, uncover that NO is produced by MECs in response to a biochemical stimulus of the ECM and plays a critical role in mammary acinar morphogenesis, whereas the lack of NO production is relevant to the malignant phenotype of breast cancer cells.

P2223

Board Number: B851

Dynamic and hierarchical reorganization of integrin-associated molecular complexes in nascent adhesions.

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An intricate network of putative molecular interactions comprises cell-matrix adhesions. Most of these interactions are inferred from co-immunoprecipitation, often using over expressed components. However, few have been demonstrated or characterized functionally in living cells, due to challenges in capturing highly regulated and transient associations that may form and evolve in adhesions. Our study uses high-resolution and image-based fluorescence fluctuation microscopy to map the formation and stoichiometry of integrin-associated molecular complexes in the nascent adhesions that populate the leading edge of migrating cells. We focus on putative integrin activating (kindlin and talin) and actin-linking (talin, vinculin and α -actinin) molecules and show that all molecules are present in nascent adhesions as soon as they are visible; however, they form integrin containing complexes hierarchically, at different times and with variable stoichiometry within the adhesion itself. These observations suggest a working model for nascent adhesion assembly, whereby transient complexes containing ~ 3 α -actinin and integrin molecules help nucleate nascent adhesions. Subsequently, integrin complexes form that contain kindlin in a 1:1 ratio, but not talin. Talin localizes to adhesions independently of the integrin-kindlin complex, and associates with vinculin in molecular complexes. However, once the nascent adhesion has formed, myosin II activity promotes talin association with the integrin-kindlin complex in a 2:1 stoichiometry, suggesting that each talin molecule cross-links two integrin-kindlin complexes. Finally, as adhesions mature into larger structures, talin increases to a 1:1 ratio with integrin and kindlin; whereas the ratio of vinculin to the other molecules continues to increase. In summary, using novel fluorescence fluctuation microscopy, we show that hierarchical adhesion assembly and reorganization accompanies adhesion formation.

P2224**Board Number: B852****Cytoskeletal integrity and dynamics regulate force mediated cell detachment.**A. Fuhrmann¹, A.J. Engler¹;¹Bioengineering, University of California, San Diego, La Jolla, CA

Integrin mediated cell adhesion is regulated by extracellular factors including divalent cations, which vary with niche. Since adhesion is ubiquitous to all adherent cells and involved in many processes, e.g. migration, quantitative information about adhesion strength is fundamental for understanding cell-ECM interactions. While it is commonly assumed that adhesion assays measure integrin-ECM bond strength, reports now show that cell material remains behind after exposure to acute shear and subsequent detachment. Here we show that many focal adhesion (FA) proteins but not actin remain behind after shear induced detachment of HT1080 fibrosarcoma cells suggesting that the cytoskeleton-FA connection disconnects during detachment more so than integrins when bound to planar substrates in the presence of divalent cations. Cytoskeletal stabilization with Phalloidin oleate (PO) did indeed increase attachment strength by 8-fold while crosslinking integrins to the substrate with DTSSP only caused a 50% increase. However, drug treatments can significantly affect cell function well before shear application. Conversely reducing the temperature only during shear application also increased attachment strength 8-fold. Even at lower temperatures, e.g. 8°C, cells leave FA proteins behind that co-localize with actin suggesting that the maximal integrin-ECM bond strength was not exceeded. The importance of temperature in stabilizing adherent cells' cytoskeleton is not well appreciated in adhesion assays with many of them operating at not further specified 'room temperature', and so we determined the dependence of attachment strength on temperature. We found that the rate of attachment strength change is most significant around room temperature (16°C – 28°C), highlighting the importance of carefully monitoring measurement temperature. Finally by observing this fracturing behavior in mouse and human fibroblasts as well as determining that it is ligand-independent, we demonstrate the ubiquity of this cytoskeleton-FA mode of detachment despite small variation in the strength of the response. Together these data demonstrate that the cytoskeleton and its dynamic coupling to focal adhesions are critically important for cell adhesion in niche with divalent cations.

P2225**Board Number: B853****Nanoscale architecture of tension generation within focal adhesions.**A.H. Mekhdjian¹, M. Morimatsu¹, A.C. Chang¹, S. Tan¹, A.R. Dunn¹;¹Chemical Engineering, Stanford University, Stanford, CA

Living cells are exquisitely responsive to mechanical cues from their surroundings. A primary means by which cells sense and transmit mechanical force is through integrins, a class of heterodimeric, transmembrane proteins that physically link the cell cytoskeleton to the extracellular matrix (ECM). The cytoplasmic domains of integrins recruit numerous proteins that collectively comprise focal adhesions

(FAs), micron-sized assemblies responsible for mechanosensing and traction force generation. However, the underlying mechanisms responsible for the spatial and temporal organization of force generation within FAs remain poorly understood. We used Förster resonance energy transfer (FRET)-based molecular tension sensors (MTSs) to directly visualize mechanical forces exerted by individual integrins. Simultaneous super-resolution imaging of MTSs and GFP-tagged cellular proteins results in maps of the force-producing structures within FAs with better than 100 nm spatial resolution, allowing us to correlate local force generation with the presence of specific integrin heterodimers and cytoskeletal proteins. We find that $\alpha\beta3$ integrin localizes to high force regions, whereas $\alpha5\beta1$ integrin localization is more diffuse. The canonical FA proteins paxillin, vinculin, talin, and α -actinin colocalize with force production to varying degrees. Surprisingly, paxillin, which is not generally considered to play a direct role in force transmission, shows a higher degree of spatial correlation with force than vinculin, talin, or α -actinin, proteins with hypothesized roles in mechanotransduction. In addition, simultaneous time-lapse imaging of either GFP-paxillin or GFP- α -actinin with MTS-measured local traction forces reveals that paxillin and tension are closely related in both space and time in assembling and disassembling adhesions, while α -actinin exhibits a more complex relationship with tension. The high degree of spatial correlation of both paxillin and $\alpha\beta3$ integrin with mechanical tension suggests that these proteins may work in concert to regulate both FA dynamics and intracellular, mechanically induced signal transduction.

P2226

Board Number: B854

Focal adhesion kinase links lamellipodial actin and nascent adhesions to the ECM through the Arp2/3 complex to regulate leading edge dynamics.

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Efficient cell migration requires proper dynamic interaction between the extracellular matrix (ECM) and the cytoskeletal network through the focal adhesion (FA) system. While FA molecules like vinculin are known to couple stress fibers through mature adhesions to the ECM, what couples dendritic actin at the leading edge through nascent adhesions (NA) to the ECM is unknown. Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that localizes to nascent and mature adhesions and plays key roles as a scaffold and a signaling molecule. FAK is thought to regulate the disassembly of mature FA, although recent evidence points to FAK playing a critical role in early adhesion events. FAK has been shown to recruit the integrin-actin linker talin to NA and also interacts with the Arp2/3 complex that nucleates the actin network in lamellipodia. Based on these results, we hypothesized a novel role for FAK in linking lamellipodial actin dynamics and NA via its interaction with Arp2/3. Using fibroblast cells derived from a FAK knockout (KO) mouse combined with high resolution DIC and TIRF microscopy, we found that FAK KO cells showed faster lamellipodial protrusion and retraction velocities as well as increased frequency in these cycles. Fluorescent speckle microscopy of actin revealed increased actin retrograde flow and

weaker engagement of the NA to the ECM marked by their sliding in the lamellipodia and a wider lamellipodia compared to controls. These defects were rescued by expression of FAK. These results implicate FAK in stabilizing NA, attenuating actin polymerization and retrograde flow at the leading edge, and delineating a lamella-lamellipodia border. To test whether FAK's role at the leading edge was dependent on its interaction with the Arp2/3 complex, we expressed a FAK mutant defective in Arp2/3 binding in FAK knockout cells. The FAK mutant was effectively recruited to NA and FA and resulted in attenuation of leading edge protrusion and actin flow velocities compared to FAK KO, indicating that FAK reduces actin polymerization and retrograde flow independent of its interaction with Arp2/3. However, compared to knockout cells expressing full length FAK, lamellipodial retraction was faster, suggesting an adhesion engagement defect. Indeed, compared to control, in cells with the FAK-Arp2/3 interaction disrupted, NAs often failed to engage to the ECM, had shorter lifetimes, and showed sliding behavior at the same rate as actin retrograde flow, similar to what was observed in the FAK KO cells. Together, these results implicate the specific interaction of the Arp2/3 complex and FAK in stabilizing the NA-ECM linkage at the leading edge of a cell which is required to attenuate edge retraction and result in overall net advance of the leading edge.

P2227

Board Number: B855

Semaphorin3A Shifts the Biphasic Relationship Between Cell Motility and Substratum Concentration Through Increased Focal Adhesion Formation.

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Cell migration plays an essential role in normal and pathological processes such as embryonic development, wound healing, and tumor metastasis. Interactions between integrin-mediated adhesions and the extracellular matrix (ECM) are important regulators of cell migration. Studies have shown that cells exhibit a biphasic relationship between cell migration speed and substratum concentration, suggesting cells experience an optimal level of cell-substratum adhesiveness to facilitate maximal cell migration (DiMilla et, al. 1993). Although these observations are well documented, mechanisms by which extracellular ligands regulate cell migration speed in response to changes in substratum concentration are not clearly understood. Semaphorin3A (Sema3A) has been found to increase integrin receptor expression in breast cancer cells (Staton et, al. 2011) as well as inhibit breast cancer cell motility (Pan et, al. 2009). Because maximal cell motility occurs when there is a balance between substratum concentration and integrin expression, we propose Sema3A alters cell adhesion dynamics to influence breast tumor cell motility on different concentrations of various ECM molecules. First, using a range of concentrations for both collagen type I and fibronectin, MDA-MB-231 cells exhibited maximal migration at intermediate concentrations. However, cells treated with Sema3A exhibited maximal cell migration at lower concentrations of both collagen and fibronectin, while Sema3A inhibited migration at intermediate and high concentrations. Second, integrin-mediated adhesion to different concentrations of either collagen or fibronectin was assessed using a spreading assay. Treatment with Sema3A resulted

in maximal cell area at lower concentrations of both collagen and fibronectin, but reduced cell area at intermediate and high concentrations of both substrata. Third, *Sema3A* increased focal adhesion formation at all concentrations of both collagen and fibronectin. These results suggest *Sema3A* shifts the optimal level of cell-substratum adhesiveness to lower concentrations of collagen and fibronectin to yield maximal cell migration. Furthermore, the data indicate *Sema3A* alters cell adhesion dynamics at intermediate and high concentrations of collagen and fibronectin, resulting in reduced cell migration speed.

P2228

Board Number: B856

PAK6 interacts with β -integrin tails and regulates integrin-mediated cell adhesion.

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PAK6 is a member of the p21-activated kinase (PAK) family, a group of six serine/threonine kinases known for their roles in modulating cell growth, survival, adhesion and motility. PAK6 was first identified as a protein that interacts with androgen receptor, and its expression has been correlated to the progression of prostate cancer. However, until recently, little more was known about the cellular roles of PAK6. Here we demonstrate that PAK6 makes a unique contribution to cell growth given that knockdown of PAK6 inhibits cell growth and this growth defect cannot be rescued by its most closely related kinases, PAK4 and PAK5, while it is rescued by re-expression of PAK6. Further, we find that PAK6 regulates cell-matrix adhesion, as knockdown of PAK6 results in increased focal adhesion size and cell spreading. We also find that PAK6 is able to directly interact with the cytoplasmic tails of β -integrins, a family of transmembrane cell adhesion receptors that link the extracellular matrix to the actin cytoskeleton. Taken together, these data support a regulatory role for PAK6 in both cell growth and cell-matrix adhesion, two basic cellular processes crucial to cancer progression.

P2229

Board Number: B857

Super-resolution microscopy reveals a role for the tetraspanin CD82 in regulating integrin molecular clustering.

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Hematopoietic stem and progenitor cell (HSPC) adhesion to the surrounding microenvironment is critical for maintaining HSPC proliferation, differentiation, and survival. However, our present understanding of

the mechanisms that regulate this adhesive interaction remains incomplete. Previously, our lab identified the tetraspanin, CD82, as a critical regulator of HSPC adhesion and homing to the bone marrow microenvironment. More specifically, we found that CD34+ cells treated with monoclonal CD82 blocking antibodies display reduced homing and engraftment in vivo. Tetraspanins, like CD82, are membrane scaffold proteins that promote the organization of membrane proteins, such as integrins, which contribute to cellular adhesion and migration. Therefore, we hypothesize that CD82 regulates HSPC adhesion by modulating integrin organization and density at the nanoscale. In order to test this hypothesis, we generated stable KG1a cells that overexpress wild-type CD82 (CD82OE). We found that the CD82OE cells display increased adhesion to fibronectin when compared to control cells. Pre-treating CD82OE cells with LDV, an $\alpha4\beta1$ specific blocking peptide, inhibited the CD82-mediated increase in adhesion, suggesting the involvement of the $\alpha4\beta1$ integrin in HSPC adhesion. In order to determine how molecular clustering contributes to HSPC adhesion, we used the super-resolution microscopy technique, dSTORM. We quantified CD82 clustering by pair-autocorrelation analysis and determined that there is an increase in CD82 cluster size in CD82OE cells when compared to the control cells. The palmitoylation of tetraspanins has been shown to regulate lateral membrane protein packing, which is critical for cell adhesion and migration. In order to test the role of CD82 palmitoylation in modulating membrane complex organization and cell adhesion, we also generated KG1a cells that overexpress a mutant form of CD82 that cannot be palmitoylated (Palm-CD82OE). As predicted, we found that the Palm-CD82OE cells display reduced cell adhesion and significantly smaller CD82 clusters than CD82OE cells. Additionally, we used the DBSCAN clustering algorithm to quantify the $\alpha4$ cluster area from our super-resolution data, finding that CD82OE cells display decreased $\alpha4$ cluster area when compared to control and Palm-CD82OE cells. Interestingly, we found an increase in the number of $\alpha4$ molecular locations per $0.01 \mu\text{m}^2$ in CD82OE cells when compared to control and Palm-CD82OE cells. Taken together, these data suggest that CD82 regulates $\alpha4$ molecular density and palmitoylation of CD82 is critical for its regulation of tightly packed $\alpha4$ clusters within the plasma membrane. Additionally, we will discuss how CD82 expression and mutation control $\beta1$ integrin activation and signaling.

P2230

Board Number: B858

Discoidin Domain Receptor 1 Impacts Myosin-Dependent Collagen Remodelling.

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The discoidin domain receptor 1 (DDR1), a tyrosine kinase receptor that is activated by type I collagen, is associated with MMP1-dependent remodelling of collagen. Our objective was to examine the contribution of DDR1 to collagen remodelling by phagocytosis, pericellular proteolysis and traction. Using mouse fibroblasts, human breast cancer cells (MCF7) and cells that do not express $\beta1$ integrin (GD25 cells), we analysed the conditioned medium and cell lysates of cells cultured on biotinylated type I collagen. With this approach we tracked the fate of exogenous collagen in different collagen remodelling processes. DDR1 expression levels were positively associated with increased collagen

phagocytosis as detected by collagen-coated bead internalization and by immunodetection using streptavidin. With the use of FITC-labeled fibrillar collagen we found that DDR1 expression was associated with higher levels of collagen compaction and fiber reorganization. In cells expressing higher levels of DDR1, there was increased pericellular collagen clearance as detected with FITC-collagen and with collagen ¾ neoepitope antibodies. Inhibition of DDR1 tyrosine kinase activity with nilotinib blocked collagen compaction, fiber alignment and phagocytosis in DDR1 expressing cells but not in DDR1 knockdown cells. Blebbistatin, an inhibitor of non-muscle myosin, did not affect the association of DDR1 and non-muscle myosin IIA in cells plated on collagen but blebbistatin strongly inhibited collagen compaction, alignment and phagocytosis. Collectively these data indicate that DDR1 associates with non-muscle myosin II to mediate collagen remodelling by mechanical reorganization and phagocytosis.

P2231

Board Number: B859

A strategy for tissue self-organization that is robust to cellular heterogeneity and plasticity.

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A combination of cell-intrinsic and extrinsic cues dictates how cell-cell and cell-ECM adhesions orchestrate the self-organization of cells into tissues. The balance between these interactions is central to the outcome of morphogenesis, tissue homeostasis, wound healing, disease progression, and the reconstructions of human tissues in vitro. Characteristics such as differential expression of cadherins and regulation of cortical tension are important cell-intrinsic parameters that influence this process. However, how extrinsic parameters, such as the physicochemical properties of the ECM, modify and relate to intrinsic interactions in the context of tissue self-organization is poorly understood. To clarify how cell-intrinsic and extrinsic cues combine to define tissue architecture in complex epithelia, we FACS-sorted human mammary epithelial cells derived from breast reduction mammoplasties into their luminal (LEP) and myoepithelial (MEP) lineages, quantified the differential mRNA and protein expression of key cell-cell and cell-ECM adhesion molecules via qPCR and flow cytometry, quantified their adhesion to each other as well as to the ECM via contact-angle measurements, translated these measurements into works of adhesion, and computationally modeled the self-organization process as a function of variable cell intrinsic and extrinsic parameters using a Metropolis-Hasting algorithm. The mathematical model predicted that MEP-ECM interactions are the dominant factor directing tissue self-organization, and that final tissue architecture is robust to perturbations to other parameters contributing to self-organization. The model also predicted that specific alterations to the balance of parameters directing self-organization could drive catastrophic breakdown of tissue architecture. To test the predictions of our model, we reconstituted compositionally and geometrically defined aggregates of luminal and basal cells

fully embedded in different ECMs using 3D cell-patterning technologies we have previously developed. This allowed us to test the consequences of specific perturbations generated with siRNA knockdowns of proteins affecting the primary cell-cell and cell-ECM interactions. We found that talin1 knockdown decreased the work of adhesion between MEPs and the ECM and promoted tissue inversion. In contrast, perturbations such as p120 knockdown in MEPs and/or LEPs decreased work of cell-cell adhesion but had no consequences on cell positioning through self-organization. Contrary to studies of early metazoan development, our results suggest that a single interaction between the basal cells and the ECM overrules cell-cell interactions and directs the self-organization of dynamic and heterogeneous epithelial tissues such as the human mammary gland.

P2232

Board Number: B860

TC21/Ras2 regulates Glycoprotein VI-FcR[gamma]-chain complex-mediated platelet activation and thrombus stability via control of Rap1 and integrin activity.

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Many Ras family small GTPases are expressed in human platelets, including Rac, RhoA, Rap, and H-/N-/R-Ras1. However, the signaling and cellular functions of most Ras family GTPases in platelets remain poorly understood. Like R-Ras1, TC21/R-Ras2 reverses suppression of integrin activation by oncogenic H-Ras; hence, TC21 may be important for integrin activation. However, a role for TC21 in platelets has not been explored. In this study, we demonstrate that TC21 is expressed in human and murine platelets and is activated in human platelets in response to agonists for the glycoprotein VI (GPVI)/FcR[gamma] ITAM-containing collagen receptor (collagen-related peptide (CRP) and convulxin). TC21 activation by GPVI required Src family kinase activity. Platelet aggregation, integrin [alpha]IIb[beta]3 activation, and [alpha]- and dense-granule secretion were significantly inhibited in TC21-deficient platelets in response to GPVI stimulation with CRP *ex vivo*. In contrast, aggregation and secretion in TC21-deficient platelets were normal in response to stimulation with 2-MeSADP (P2Y receptor agonist), AYPGKF (PAR4 receptor agonist) and fucoidan (CLEC-2 receptor agonist), indicating that the function of TC21 in platelets is GPVI/FcR[gamma]-ITAM-specific. CRP-induced phosphorylation of Syk, PLC[gamma]2, Akt, and ERK were substantially inhibited in TC21-deficient murine platelets. Stimulation with CRP, but not with other agonists, induced enrichment of TC21 at the plasma membrane in human platelets, and TC21 was required for GPVI-induced activation of Rap1b, indicating Ras small GTPase crosstalk. Using a thrombosis model with FeCl₃-induced injury to the carotid artery, we observed a delay in initial thrombus formation in TC21-deficient mice compared to WT controls; furthermore, thrombi in TC21-deficient mice were

unstable, leading to prolonged time to thrombotic occlusion. Tail clip bleeding times showed similar effects in these mice. Thus, TC21 plays an essential role in GPVI/FcR[gamma]-mediated platelet activation and is important for thrombus stability *in vivo* via control of Rap1b and integrins.

P2233

Board Number: B861

Defining Phosphatidylinositol Phosphate 5-Kinase Igamma Function in Cell Migration and Anoikis Evasion.

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Type I phosphatidylinositol-4 phosphate 5-kinases (classified into PIPKIalpha, PIPKIbeta and PIPKIgamma) generate phosphatidylinositol 4,5-bisphosphate (PIP2), a lipid signaling molecule that regulate diverse cellular functions, including cell cycle progression, cell survival, endosomal trafficking and cell migration. PIPKIgamma is overexpressed in triple-negative breast cancer tissues and inversely correlates with breast cancer patient survival. In order to define the PIPKIgamma function in tumor progression, we interrogated the PIPKIgamma function in the context of epithelial cells transitioning into the mesenchymal cells, as EMT provides an important platform for understanding how normal cells acquire different traits crucial for tumor progression. Mammary epithelial cells transitioning into mesenchymal cells by TGFbeta1 treatment or by culturing in extracellular matrix proteins displayed significantly increased expression of PIPKIgamma and its focal adhesion targeting variant, PIPKIgamma2. In these cells and other breast cancer cells, PIPKIgamma/PIPKIgamma2 were required for activation of phosphoinositide signaling in response to activation of cells with growth factors and extracellular matrix proteins. During epithelial mesenchymal transition, PIPKIgamma2 lose its association with epithelial protein (E-cadherin) and gain increased association with different pro-migratory molecules, such as talin, moesin, N-cadherin, Src and IQGAP1. Consistently, the loss of PIPKIgamma2 and its associating partners dramatically impaired cell migration. Furthermore, PIPKIgamma2 and talin interaction and their collaborative function were required for providing the signaling inputs from the extracellular matrix proteins and growth factors to maintain the migratory and mesenchymal phenotypes of epithelial cells transformed into mesenchymal cells.

Anoikis evasion and the ability to grow in anchorage-independent condition define one of the key properties of metastasizing tumor cells. PIPKIgamma and PIPKIgamma2 shows increased expression in anoikis resistant cancer cells during suspension culture and is crucial for sustaining phosphoinositide signaling. The loss of PIPKIgamma and PIPKIgamma2 dramatically impaired the ability of different tumor cells to grow in anchorage-independent condition. These indicate the diverse pro-tumorigenic function of PIPKIgamma and PIPKIgamma2 in cancer.

P2234**Board Number: B862****Phorbol ester alters cell migration of lung adenocarcinoma on laminin-511.**K. Ikari¹, S. Fujii², F. Katagiri², K. Hozumi², M. Nomizu², Y. Kikkawa²;¹School of Pharmacy, Tokyo Univ Pharm/Life Sci, Tokyo, Japan, ²School of Pharmacy, Tokyo Univ Pharm/Life Sci, Hachioji, Japan

Progression of tumor cells results from a multi-step process of carcinogenesis that involves not only sequential genetic alterations but also frequent chemical exposures. Epithelial cells of several organs such as skin and lung are subject to frequent carcinogens and pathogens. Tumor-initiated cells seem to be also transitioned to malignant tumors by exposure to tumor-promoting agents. Although precancerous cells of epithelium stably anchor to basement membrane, malignant tumors pass through it to achieve metastasis. Of basement membrane components, laminin-511 ($\alpha 5$, $\beta 1$, $\gamma 1$; LM-511) has been found to be a major isoform in many adult basement membranes. Several studies have also shown that LM-511 promotes not only cell adhesion but also tumor cell migration. Thus, LM-511 can be viewed as a double-edged molecule in normal and tumor cells. This is because tumor cells that arise from chemical exposures could alter cell adhesion to LM-511. In this study we examined the effects of potent tumor-promoting agent on lung adenocarcinoma adhering to LM-511. 12-O-tetradecanoylphorbol-13-acetate (TPA) is a phorbol ester that links to the modulation of protein kinase C in epithelial cells. The cells readily attached to LM-511 and Fibronectin (Fn) and spread on the substrata. After adhering to the substrata, the cells were treated with TPA. TPA-treated cells exhibited cuboidal shape on LM-511. On the other hand, the morphology of the cells on FN was flatter or spindle. Flow cytometric analysis showed that the treatment of TPA on A549 cells did not influence the expression of laminin receptors such as integrins and lutheran. Cell attachment assay showed the attachment of TPA-treated A549 cells to LM-511 was weaker than that of control cells, suggesting that the treatment of TPA modulated the binding of laminin receptors. We also examined the cell migration of TPA-treated A549 cells on LM-511. Cell migration was monitored using time-lapse video microscopy. The treatment of TPA promoted cell migration on LM-511 but not FN. We conclude that tumor cell migration on LM-511 requires that the treatment of TPA modulates cell-attachment through the laminin receptors. We suggest that tumor promotion is involved in a transition from static to migratory cell behaviors.

P2235**Board Number: B863****An anti-human Lutheran phage antibody isolated from hepatocellular carcinoma phage library inhibits cell migration on laminin-511.**Y. Kikkawa¹, Y. Enomoto², K. Ikari³, F. Katagiri¹, K. Hozumi¹, M. Nomizu¹, Y. Ito²;¹School of Pharmacy, Tokyo Univ Pharm/Life Sci, Hachioji, Japan, ²Graduate School of Science and Engineering, Kagoshima University, Kagoshima, Japan, ³School of Pharmacy, Tokyo Univ Pharm/Life Sci, Tokyo, Japan

The Lutheran glycoprotein (Lu), also known as basal cell adhesion molecule (B-CAM) is an Ig superfamily (IgSF) transmembrane receptor for laminin $\alpha 5$, a subunit of laminin-511 that is a major component of basement membranes in various tissues. So far the molecule has been studied primarily in the contexts of blood group antigens and sickle cell disease. B-CAM, a splice variant of Lu, has the same N-terminal extracellular domain as Lu, but it lacks the C-terminal 40 amino acids of the cytoplasmic tail which carries an SH3 binding motif and potential phosphorylation sites that could be involved in intracellular signaling pathways. B-CAM was also identified as an up-regulated antigen in carcinoma, suggesting its involvement in tumor progression. Our previous study showed that although Lu/B-CAM is not present in normal hepatocytes, the expression of Lu/B-CAM is significantly increased in hepatocellular carcinoma cells (HCCs). In this study we isolated thirteen phage antibodies against human Lu/B-CAM from phage library of peripheral blood of HCC patients, suggesting that HCC patients produced autoantibody against endogenous Lu/B-CAM. The RNA of peripheral blood cells was provided from Dr. Hui Kam Man, Natl Cancer Center, Singapore. To characterize the phage antibodies, we determined the epitopes of phage antibodies on Lu. The extracellular domain of Lu contains five IgSF domains, D1-D2-D3-D4-D5. The epitope of A7 phage antibody was localized in D5 domain. The other phage antibodies recognized to D2 domain that is recognized with function blocking mouse monoclonal antibody. Not only C7 phage antibody recognizing to D2 domain inhibited the binding of Lu to ligand, it also prevented tumor migration on laminin-511 (LM-511). However the C7 scFv purified from the periplasm fraction of bacteria could not exhibit the inhibitory effects, indicating that scFv form was not enough to inhibit spatially the binding of Lu to LM-511. Furthermore we identified the amino acid residues to form the epitope recognized by C7 phage antibody. The chimeric mutant study using human and mouse Lu showed that it was formed with amino acid sequences from Ala²³⁸ through His²⁵⁷. The mutagenesis study showed that Arg²⁴⁷ is necessary for forming the epitope. C7 phage antibody and its epitope may be useful for developing drugs to prevent HCC progression.

P2236**Board Number: B864****Integrin-integrin Crosstalk Suppresses Fibroblasts Adhesion via PI3K signaling.**K. Hozumi¹, C. Fujimori², F. Katagiri¹, Y. Kikkawa¹, M. Nomizu¹;¹School of Pharmacy, Tokyo Univ Pharm/Life Sci, Hachioji, Japan, ²Tokyo Univ Pharmacy/Life Scis, Hachioji, Japan

Various cell surface receptors such as growth factor receptors, GPI anchored receptors, syndecans, and different subtypes of integrin are synergistically related to integrin mediating cell attachment. Several studies has been reported about the crosstalks between different subtypes of integrin and revealed that combination specific integrin-integrin crosstalk is critical on the various biological functions. Here, we focused on various crosstalks of two different subtypes of integrins using the unique integrin binding peptides conjugated polysaccharide (chitosan or alginate) matrix. Six different integrin binding peptides, FIB1 (integrin $\alpha v \beta 3$), EF1zz (integrin $\alpha 2 \beta 1$), 531 (integrin $\alpha 3 \beta 1$), CS1D (integrin $\alpha 4 \beta 1$), and A2G10 (integrin $\alpha 6 \beta 1$), were mixed and conjugated on the polysaccharide matrix with five different mixture ratios (9:1, 4:1, 1:1, 1:4, 1:9). Human dermal fibroblast (HDF) attachment activities to the 20 different peptide-chitosan matrices were different depending on the peptides and mixture ratios. Ten different pair of mixed peptides-chitosan matrices did not show any special variation by mixture of peptides, and the mixture of FIB1/A2G10, FIB1/CS1D, and EF1zz/CS1D relatively promoted HDF attachment by mixing of two peptides. Interestingly, although EF1zz:531=1:4 showed significant decreasing of cell attachment, but other mixture ratios between EF1zz and 531 did not indicated any differences on both chitosan and alginate matrices, respectively. When we focused on the signal transduction of the EF1zz/531 at 1:4 mixtures, Y397 phosphorylation of FAK was relatively decreased, however Y514 phosphorylation of Src was not. Next we observed the effect of various activator and inhibitor on HDF adhesion to the EF1zz/531-alginate matrices. Integrin activation by TPA or function antibody TS2/16 and PI3K inhibitor of Wortmannin rescue the suppression of HDF attachment at EF1zz:531=1:4, whereas PKC inhibitor of Go 6976 did not affect on that suppression. Further, PKA inhibitors accelerate the suppression of HDF attachment, these suggesting that this suppression was mediated by PI3K signaling through the activation of integrin. From these results, specific integrin-integrin crosstalk promotes or suppresses the cell attachment, and mixed peptide-polysaccharide matrix is useful tool to understand the integrin functions.

P2237**Board Number: B865****Hypoxia enhances differentiation into invasive extravillous trophoblast following BMP4 treatment of human embryonic stem cells.**A.K. Wakeland¹, M. Moretto Zita², F. Soncin², Y. Li², M. Horii², M. Parast²;¹Biomedical Sciences, University of California San Diego, San Diego, CA, ²Pathology, University of California San Diego, San Diego, CA

Trophectoderm lineage specification and the bulk of placental growth occur under low oxygen tension. Cytotrophoblast (CTB) are epithelial stem cells in the placenta, which differentiate further into two lineages: hCG-secreting villous syncytiotrophoblast (STB), or invasive extravillous trophoblast (EVT). Proper differentiation into these two lineages is important for appropriate placental function and fetal growth *in utero*. We have recently shown that human trophoblast lineage specification and terminal differentiation can be modeled *in vitro* using human embryonic stem cells (hESC). Following treatment with bone morphogenetic protein 4 (BMP4), hESC first differentiate into proliferative CTB, and subsequently into STB and EVT. Differentiation into EVT is characterized by changes in integrin expression as well as expression of matrix metalloproteinases (MMPs), confirming the importance of extracellular matrix (ECM) remodeling in this process. We tested the effects of different ECM and oxygen tension, first on differentiation of primary CTB isolated from first trimester placental tissues. We found that decreasing oxygen levels resulted in impaired differentiation into villous STB based on hCG secretion. Conversely, we found that low oxygen tension directed trophoblast differentiation into invasive EVT lineage, based on increased surface expression of HLAG and integrin alpha-1, as well as secretion of MMP2, which are specific to invasive EVT. EVT differentiation was most optimal when cells were plated on fibronectin, and least optimal on laminin. Similar results were obtained when hESC-derived CTB were induced to terminally differentiate, with cells optimally differentiating into EVT when plated on fibronectin and cultured under low oxygen (2%). In addition, hypoxia appeared to accelerate trophoblast lineage specification, with a 20% increase in percentage of EGFR+ CTB after only 2 days of BMP4 treatment under hypoxia. These results show the importance of both ECM and oxygen tension in EVT differentiation, but more importantly, confirm the utility of BMP4-treated hESCs as a model that correctly recapitulates primary EVT differentiation. This is particularly critical as hESC can thus provide a ready alternative to first trimester CTB for the study of EVT differentiation, a process that is highly important to pregnancy success. We are currently testing the role of an intact hypoxia-inducible factor (HIF) complex in both establishment and terminal differentiation of the trophoblast lineage, and hypothesize, based on mouse models of placentation, that it plays a pivotal role in this process, but particularly in differentiation of invasive EVT.

P2238**Board Number: B866****The integrin beta3 tail controls switching between Rac-driven collective migration and Rho-stimulated cell scattering.**A. Jimenez Orgaz*¹, E. Anthony*², H. Truong¹, I. van den Bout¹, I. Kuikman¹, P. Secades¹, A. Sonnenberg*¹, C. Margadant*²;¹Department of Cell Biology, Netherlands Cancer Institute, Amsterdam, Netherlands, ²Department of Molecular Cell Biology, Sanquin Research, Amsterdam, Netherlands

Expression of the fibronectin-binding integrin alpha5beta1 in epithelial cells induces cell scattering, RhoA activity, random single-cell migration, and a switch to a fibroblast-like phenotype. In contrast, the alternative fibronectin-binding integrin alphavbeta3 supports Rac activation, and stimulates collective, directional migration with intact cell-cell contacts. We tested the importance of cytoplasmic domains in the beta3-subunit on cell behaviour. Disruption of the talin-binding NPxY and/or the kindlin-binding NITY motif reduced initial cell adhesion and cell spreading, which was recapitulated by depletion of talin-1 or kindlin-2, respectively. Downstream of cell adhesion, deletion of the NITY motif or Y>A mutation induced an 'alpha5beta1'-like phenotype, consisting of cell scattering, Rho activity, and random, single-cell migration. These effects are independent of kindlin-2 or Src, but entirely dependent on Rac, and are recapitulated by either the expression of dominant-negative Rac mutants, or constitutively-active Rho. Similarly, the phenotype of cells that express beta3-NITY>A mutants is reversed by overexpression of constitutively-active Rac, or by the inhibition of Rho. Thus, the NITY motif of beta3 controls the balance between Rac-driven collective migration and Rho-stimulated cell scattering, in a kindlin- and Src-independent manner.

Bioengineering of Cell–Matrix Interactions**P2239****Board Number: B867****Minimal matrix model with scar-like fractal heterogeneity reveals a mechano-sensitive repressor in stem cell plasticity.**P.P. Dingal¹, A. Bradshaw¹, S. Cho¹, J. Swift², M. Raab³, A. Buxboim⁴, D.E. Discher^{1,5};¹Chemical and Biomolecular Engineering, University of Pennsylvania, Philadelphia, PA, ²University of Pennsylvania, Philadelphia, PA, ³UMR 144, Institut Curie, Paris, France, ⁴Hebrew University of Jerusalem, Jerusalem, Israel, ⁵Molecular and Cell Biophysics Lab, University of Pennsylvania, Philadelphia, PA

Scarring in higher animals has an adverse and sustained effect on tissue, but the diversity of cell types in a scar complicates understanding of the factors affecting cell phenotype. Here, co-polymerization of collagen-I with polyacrylamide was used to produce a minimal matrix model of a scar in which fractal fiber bundles segregated heterogeneously to the subsurface of an inert hydrogel. Matrix stiffens locally

– as in scars – while allowing separate control over adhesive ligand density. Mesenchymal stem cells (MSCs) that are now being used in various tissue repair contexts respond to the model matrices with phenotypes often seen in scars: they polarize (more so than on rigid plastic) and slowly increase expression of ‘scar marker’ smooth muscle actin (SMA). MSCs also traffic to injured sites *in vivo*, and here they polarize and ‘durotax’ towards scar-like regions of matrices where a subset of stiffness-sensitive proteins respond within hours. Over days, a repressor of SMA transcription, NKX2.5, translocates from nucleus to cytoplasm in response to matrix rigidity, suggesting a mechanism for SMA de-repression. Expression of nuclear-localized mutants of NKX2.5 indeed overrides matrix-rigidity sensing with inhibition of cell spreading and SMA. MSCs cultured for weeks or longer on rigid substrate reinforce a ‘mechanical memory’ by down-regulating NKX2.5, thereby stabilizing an SMA-high, scar-like phenotype.

P2240

Board Number: B868

The Interplay of Duro- and Hapto-taxis in Regulating Stem Cell State.

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Anchorage dependent cells can sense and respond to extracellular matrix (ECM) properties, e.g. stiffness and ligand density, but gradients of these cues are often found *in vivo* via normal tissue variation or pathological conditions, such as the post-infarct myocardial scar which is several fold stiffer as well as compositionally different from healthy tissue. Scar tissue and fibrotic tissue are often characterized by the presence of excess matrix proteins such as collagen and fibronectin due to increased deposition and decreased degradation, forming gradients adjacent to healthy tissue. We have previously shown that cell migration can be regulated by substrate stiffness *in vitro*, but stiffness-directed migration, i.e. durotaxis, may be additionally accompanied *in vivo* by haptotaxis, i.e. migration up a ligand concentration gradient. To better understand how corresponding and opposing gradients regulate cell migratory behaviors, tunable gradients of stiffness and collagen concentration in a polyacrylamide hydrogel system were constructed using density gradient multilayer polymerization, a technique that utilizes phase separation between liquids of varying density to create layers of distinct composition. Stiffness gradients were obtained by varying the monomer to crosslinker ratio, or by applying a UV photomask in order to activate a photoinitiator gradient. Ligand gradients were obtained by varying the concentration of acrylic acid, thus sequentially varying the density of EDC/NHS linker, in each hydrogel layer; this creates a polyacrylamide substrate with a surface density gradient of covalently bound collagen. Stiffness and ligand gradients were overlaid to investigate the effect of corresponding and opposing haptotactic and durotactic gradients on fibroblast and mesenchymal stem cell (MSC) migratory behavior. Fibroblasts tended to haptotax towards regions of increased collagen concentration and durotax towards stiffer regions of the substrate. While MSCs also underwent durotaxis, surprisingly they

did not haptotax. These data suggest that fibroblasts residing in a tissue with such gradients may be more responsive than MSCs, which must home to the tissue from marrow to aide in its repair.

P2241

Board Number: B869

Malignant melanoma cells dynamically construct a tumor biofilm that promotes survival in response to drug treatment and hypoxia.

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Cutaneous melanoma cells secrete and assemble a myriad of extracellular matrix (ECM) molecules into dense fibrillar and globular networks. This process is dynamic, mimicking biofilm formation by bacteria in response to nutrient deficiencies, antibiotic treatment and oxidative stress. We hypothesize that the melanoma 'tumor biofilm' is similarly adaptive and protective in response to external insults, including pharmaceutical intervention and hypoxic stress. Using quantitative proteomics, we identified the components and determined the temporal evolution of the tumor biofilm. Isogenic melanoma lines were cultured in 3D to recapitulate tumor morphology and interrogated for the establishment of a de novo tumor microenvironment as a function of dimension, oxygen tension and drug treatment. We recreated an established tumor and drug regimen using a modified 3D soft agarose assay to evaluate anchorage independent proliferation. Modulation of the observed tumor biofilm in culture was monitored using confocal microscopy, protein expression analysis and immunofluorescence, and its physiological impact on primary tumor establishment was examined in a mouse subcutaneous injection model. Fibronectin was found to be one of the key architectural components, regulating drug efficacy for a broad spectrum of drug therapies. Stable cell lines engineered to secrete minimal levels of fibronectin became sensitive to cisplatin, BRAF, and ERK inhibition as clonally derived 3D tumor aggregates, emphasizing the importance of 3D architecture in tumor proliferation. Reduced levels of tumor-generated fibronectin also impeded successful tumor formation in mice. We reason that this architectural complexity provides the degree of plasticity and flexibility that allows for better colonial adaptability and survival in response to external perturbations, such as hypoxic stress and drug treatments

P2242**Board Number: B870****Cell-bioactive glass scaffold interactions: The role of substrate nano-structure.**T.J. Kowal¹, T. Chokshi¹, H. Jain², M.M. Falk¹;¹Biological Sciences, Lehigh University, Bethlehem, PA, ²Material Science and Engineering, Lehigh University, Bethlehem, PA

It is not well understood how cells of the human body can interact with biologically active scaffolds used to guide tissue regeneration, especially on the nano-scale level. In order to design the ideal material for the regeneration of different tissues, we must first acquire a comprehensive understanding of the fundamental mechanisms that allow cells to not only attach to these bioscaffold materials, but to also take cues from them as to what tissue should be regenerated.

Here we present evidence of the ability of MC3T3-E1 pre-osteoblasts to detect differences in nano-structure on two different substrates. The first substrate, a bioactive glass with a simple composition of 30 mol%CaO – 70 mol%SiO₂ contains both nano- and macro-pores. The only variable between samples of this type of substrate is the nano-pore size (3.7 nm vs. 17.7 nm), which is measured using BET (nitrogen adsorption analysis), while total surface area is kept constant. The second substrate, a bioactive glass with a more complex composition of 24.4 mol%Na₂O–26.9 mol%CaO–2.6 mol%P₂O₅–46.1 mol%SiO₂ called 45S5 Bioglass[®] (Hench et al., 2006; J Mater Sci: Mater Med), varies in the nano-structure due to phase separation, resulting in either a spinodal interconnected morphology or a droplet nucleation morphology as determined by SEM analysis (Golovchak et al., 2014; Acta Biomaterialia). Cellular response to either substrate was measured through quantification of initial adhesion to the surfaces using immunofluorescence analyses. On the glass samples varying in nano-pore size, we observed that cells preferred the smaller nano-pores (Wang et al., 2013; Tissue Engineering, PartA). Additionally, a clear preference was observed using the glass that differed in nano-structure in that more adherence was observed on the spinodal interconnected morphology than the droplet nucleation morphology. In the case of both substrates, cells showed a significant preference to one of the nano-structures, indicating that remarkably cells are able to sense morphological details of the substrates that are app. 1000 times smaller than the cells themselves. Research performed in our laboratories aims to uncover the underlying principles that allow cells to respond to such nano-scaled substrate details, allowing to engineer superior tissue regenerative materials.

P2243**Board Number: B871****Cellular response to substrate curvature is cell-type dependent.**A. Yip¹, K. Chiam^{1,2};¹Bioinformatics Institute, A*STAR, Singapore, Singapore, ²Mechanobiology Institute, National University of Singapore, Singapore, Singapore

Experiments have shown that cells experience and respond to a variety of mechanical forces in physiological conditions, such as those caused by neighbouring cells as well as those due to geometrical constraints of the extracellular matrix. For example, it has been shown that cells can sense and respond to the rigidity of the extracellular matrix, topographical features of the extracellular matrix, and imposed shear stresses. However, little is known about the effects of substrate curvature on cell behavior. In this study, we fabricated substrates of a range of rigidities with cylindrical grooves of varying radii of curvature. We then characterized the morphology of both mesenchymal NIH 3T3 fibroblasts and A549 human lung adenocarcinoma epithelial cells on these substrates. The radius of curvature of the cylindrical grooves were varied from dimensions comparable to a single cell diameter (20 μm) to dimensions of approximately ten cell diameters wide (250 μm). We found that as the radius of curvature decreased, mesenchymal cells became increasingly elongated with a larger proportion of the cells orienting along the long axis of the cylindrical grooves. In contrast, epithelial cells do not show any variation in cell elongation or orientation when the radius of curvature is changed. We then investigated the role of cadherins in the epithelial monolayers in contributing to this effect by the addition of EDTA. Finally, we developed a quantitative model to explain these results. In summary, our results suggested that mesenchymal and epithelial cells respond differently to substrate curvature, and could provide new insights into the process of epithelial to mesenchymal transition.

P2244**Board Number: B872****Low adhesive scaffold collagen, inducing spheroid formation, promotes the osteogenic differentiation.**S. Kunii¹, E. Yamamoto¹, Y. Horiuchi², H. Ito¹, K. Morimoto¹;¹Biology-Oriented Science and Technology, Kindai University, Kinokawa, Japan, ²Kindai University Faculty of Medicine, Kindai University, Osakasayama, Japan

[Background] Collagen has biocompatibility and biodegradability with tissue or organs, therefore, collagen is the most promising material for tissue engineering. In particular, the binding of collagen to specific cells is considered an essential function to develop scaffolds. However, in some cases the binding inhibits the cell motility. We succeeded in developing low adhesive scaffold type I collagen (LASCOL) by enzyme treatment (patent pending). In this study, we report that LASCOL markedly facilitates osteogenic differentiation of rat marrow mesenchymal stem cells (rMSCs). Furthermore, we investigated the effects of bone wound healing by implanting LASCOL in a defect of rat tibia. [Methods] We obtained

LASCol by the enzyme treatment of pig type I collagen. The culture dish was coated with LASCol or pepsin-treated collagen (PepCol). Subsequently, rMSCs were cultured on each coated-dish with osteogenic basal medium. We observed the morphology of rMSCs by using a phase-contrast microscope. To evaluate osteogenic differentiation, we quantified the mineralization by SEM-EDX. Furthermore, we transplanted each collagen material into the defect of rat tibiae (ϕ 2.5 mm critical-sized defect). After 15 days, bone regeneration efficiency of each implant treatment was evaluated by histological observation of tibia horizontal section with HE stain. Osteocalcin in each serum was measured by ELISA kit. [Results] Rat MSCs formed spheroid bodies by culturing on the LASCol coated dish. We observed that spheroids adhered to LASCol scaffold. Each cell of spheroid highly expressed alkaline phosphatase activity. The mineralization of rMSCs was significantly promoted by culturing on LASCol. Only spheroid cultured on LASCol showed the activity of mineralization by staining with Alizarin red S reagent. Similarly, the P and Ca contents markedly increased. Interestingly, the results *in vivo* demonstrated that LASCol graft induces bone regeneration of rat tibia and is more bioabsorbable material than PepCol. Osteocalcin in serum of LASCol implant rats (194 ng/mL) was two-fold higher than that of PepCol (105 ng/mL). [Funding] This work was supported by Adaptable and Seamless Technology Transfer Program through target-driven R&D, Japan Science and Technology Agency (AS2414037P to K.M.).

P2245

Board Number: B873

Quantitative Investigation of Differences in Differentiation of Reactive and Non-reactive Astrocytes in Response to Nanophysical Cues.

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Recent studies of cell responses to elasticity, surface roughness, surface polarity and nano-patterning suggest that controlling aspects of the nanophysical environment holds potential for inducing preferential differentiation of reactive astrocytes into non-inhibitory pathways. In the present studies [1,2], these cues were presented by an implantable electrospun polyamide nanofibrillar scaffold that has demonstrated promising wound healing properties including *in vivo* mitigation of astrocytic scarring. Only external cues were used to trigger cell responses. Four physical properties of the nanofibrillar polyamide versus poly-L-lysine (PLL) glass, PLL Aclar and Aclar culture surfaces were considered potentially directive: nanoscopic elasticity, work of adhesion, surface roughness, and macroscopic surface polarity. These were quantified using AFM and contact angle measurements. Differentiation of untreated versus reactive-like (dBcAMP-treated) astrocytes was investigated using AFM, super-resolution microscopy and confocal laser scanning microscopy z-series, with 50-cell minimum protein quantification for major neuro-reactivity markers: glial fibrillary acidic protein (GFAP), Beta tubulin and neuro-inhibitory chondroitin sulfate proteoglycans (CSPGs) and also Rho GTPase cytoskeletal upstream regulators Cdc42 (filopodia), Rac1 (lamellipodia), and RhoA (stress fiber/stellation).

The physical property differences of the culture environments triggered statistically significant (ANOVA with pairwise comparisons by Tukey's test, P

P2246

Board Number: B874

Label-free quantitative analysis of cancer cell dynamics in topologically and mechanically defined 3D matrices.

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Interactions between cells and their extracellular microenvironment influence multiple aspects of cellular functions and fate decision in physiological and pathological processes. Cancer cells have a highly distinctive ability to adapt and communicate to their microenvironments. The properties of extracellular microenvironments, namely topology, mechanics as well as composition, regulate the development and progression of cancer. Moreover, migration and invasion of cancer cells through extracellular microenvironments are crucial steps for metastasis. A deep understanding on underlying mechanisms is still hampered by the complexity of the mechanical regulation of cell behavior as well as the heterogeneity of relevant cell populations. Understanding how extracellular matrix properties, regulate cell behavior at single cell level would provide potential strategies in approaches to fight cancer.

In this work, we reveal cellular characteristics of melanoma cells, including migration pattern, proliferation and differentiation features, under exogenous influence of ECM topology and mechanics. In order to mimic three-dimensional (3D) *in vivo* like microenvironments, we reconstruct 3D collagen matrices with well-defined pore size, fibril diameter and stiffness. The topological and mechanical parameters are varied by adjusting polymerization conditions and were quantified by image analysis tools and force spectroscopy, respectively. The impact of biophysical parameters of 3D matrices on single cell behavior was analyzed using automated quantitative label-free 3D single cell tracking. First cell experiments reveal distinct migration characteristics of different melanoma cell types in correlation with 3D matrix properties. The matrix-specific migration characteristics of melanoma cells allowed a mechanistic understanding of invasive migratory behavior and prediction of cell fate. Furthermore, we observed that fibril orientation of collagen matrices contribute in guiding migration of melanoma cells in a size-dependent manner.

Summarizing, our work emphasizes the complex relationship between structural-mechanical properties of the extracellular matrix and invasive migratory behavior of cancer cells and provides new approaches for studying heterogeneous populations of primary cells in a biomimetic 3D microenvironment.

P2247**Board Number: B875****Oral administration of Nano-sized avian eggshell membrane is digested, absorbed, distributed to various organs, and contribute to support good health.**E. Ohto-Fujita¹, M. Shimizu¹, N. Nogawa², H. Eguchi², K. Ijiri², Y. Hasebe³, Y. Atomi¹;¹Div. of Material Health Sci., Dept. of Technol., Tokyo University of Agriculture and Technology, Tokyo, Japan, ²Radioisotope Center, The University of Tokyo, Tokyo, Japan, ³Almado Inc., Tokyo, Japan

Eggshell membrane (ESM) is a double-layered insoluble sheet locates between eggshell and egg white and works as a scaffold for biomineralization to fabricate egg shell (Rose and Hincke, 2009). ESM mainly consists of various extracellular matrix (ECM) materials necessary for maintaining homeostasis against stimuli from environment during embryogenesis and development until chick birth. The membrane is formed in the isthmus of the hen's oviduct before shell mineralization and egg laying. Recently ESM containing cosmetics and supplements are on the market worldwide based on the evidence from traditional folk medicine in Asian countries. Until last year we have reported that topical application of alkaline-solubilized avian eggshell membrane (ASESM) improved gene expression pattern to younger skin after 10 days, like increases of gene trio like type III collagen, decorin, and MMP2 on the skin of hairless mice, and increased elasticity of human skin after 3 months. In the present study digestion, absorption and disposition after single oral administration of tritium-labeled ESM were examined using C57BL/6J mice was examined. Tritium derived from peroral labeled ESM showed peak in blood after 6 hour and later disposed into various tissues including liver. Similar to topical effect to improve dermis ECM condition non-labeled nano-powdered ESM peroral administration showed to improve liver ECM environment, such as significant decreased gene expression of type I and III collagen, which increases with liver fibrosis, compared with control. Chicken ESM may work as materials digested during embryogenesis to contribute the development as well as filter, shelter and as a scaffold for biomineralization. ESM has an ability to keep ECM environment of various differentiated tissue to be young through unsolved mechanism.

P2248**Board Number: B876****Avian eggshell membrane as an effective ingredient for health supplement preventing liver fibrosis by suppressed collagen gene expression.**M. Shimizu¹, E. Ito¹, A. Atomi¹, E. Ohto-Fujita¹, Y. Hasebe², Y. Atomi¹;¹Div. of Material Health Sci., Dept. of Technol., Tokyo University of Agriculture and Technology, Tokyo, Japan, ²Almado Inc., Tokyo, Japan

Avian eggshell membrane (ESM) has long been utilized as a Chinese medicine for recoveries from burns, injuries, and wound. Even nowadays, Japanese Sumo wrestlers use it for wound healing. Extra-fine powdered ESM is on market and used as a supplement that improve various health problems. ESM has a

fibrous network mainly comprised of extracellular matrix (ECM) proteins such as type I, V, and X collagens. We have found that water-soluble alkaline-digested form of ESM can stimulate type III collagen, decorin, and MMP2 expression in human dermal fibroblast cells (Ohto-Fujita et al, 2011). As a part of understanding the whole-body health benefit of ESM, here we report the molecular basis of ESM supplement on liver. Either 6 weeks old male Hairless mouse (Hos HR-1) or Wister rat were used after one-week acclimation. Mice were fed ad libitum and additionally let them take either low or high-dose nano-sized powdered ESM supplement daily for 7 days. Rats were fed ad libitum and ESM was administered by gastrointestinal intubation for 14 days. Total RNA from liver was prepared and quantitative real time PCR analysis was performed. Results showed that down-regulated collagen expression (both type I and III collagen for mouse significantly and type I collagen for rat) and up-regulated HSP70 (mouse at high dose). These results were comparable to the report on gene expression in rat liver (Jia, et al., 2013). Since 45% of deaths in the United States can be attributed to fibrotic disorders (Nature Rev Immunol, 2004), suppressed expression of type I collagen is useful evidence for health. Previous and current data shows ESM is an ECM conditioner and mediate tissue rejuvenation with unsolved mechanism.

P2249

Board Number: B877

Anisotropic effects of three-dimensional confined geometries on cell growth and cell-derived extracellular matrix.

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Extracellular matrix is a complex, 3D mesh of proteins, proteoglycans, and bound growth factors that provides mechanical and biochemical environmental cues for cellular growth. Previous work in our lab showed that matrix produced and assembled by human fetal lung fibroblasts (WI38) provided a morphogenic stimulus to endothelial cells, inducing both matrix remodeling and tubulogenesis. Microvascular development in a natural complex scaffold material is an attractive platform for regenerative therapies.

The current study tests the hypothesis that confined 3D geometries induce early anisotropy in WI38 growth, actin organization, and matrix deposition. Using microfabrication and photolithography, we created arrays of PDMS wells that included three rectangular shapes with increasing aspect ratios (1:2, 1:4, and 1:8), and corresponding paired squares with the same depth, total surface area, and total volume. Microcontact printing of bovine serum albumin (BSA) was used to block fibroblast adhesion to

the top surface of the PDMS block. The orientation of W138 stress fibers and fibronectin fibrils were examined 1 and 2 days after seeding using laser scanning microscopy and a custom routine that was developed for quantitative autocorrelation analysis (MATLAB (MathWorks)). Anisotropic actin filament organization and fibronectin fibril deposition were observed in the rectangular shapes. In these shapes, cells showed increasing alignment with the long axis as the aspect ratio of the rectangles increased. Actin filaments of cells cultivated in rectangular wells with aspect ratios of 1:4 and 1:8 exhibited anisotropy scores that were significantly higher than those grown in the corresponding squares or in lower aspect ratio rectangular wells. Similar trends were seen with fibronectin. These data suggest that shape-specific effects of 3D confinement on fibroblasts influence actin stress fiber alignment and matrix deposition.

Latrunculin A (0.05uM) decreased cytoskeletal and matrix anisotropy at all but the highest (1:8) aspect ratio. Cells treated with nocodazole (0.02uM) did not exhibit alignment of the microfilament or microtubule cytoskeletons or of cell-assembled matrix with anisotropic spatial confinement. In contrast, treatment with Y27632 (4uM) did not inhibit the development of cytoskeletal or matrix anisotropy. These data implicate both actin and microtubule polymerization, but not ROCK-dependent acto-myosin interactions, in anisotropic responses of cellular growth and matrix assembly to spatial confinement. Next phases of this project include definition of the effects of 3D, confined geometries on blood vessel growth and orientation in natural matrix.

Regulation of Aging

P2250

Board Number: B879

Defective development of Natural Killer cells during aging.

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Natural Killer (NK) cells are important in combating both infections and cancer. Using Ectromelia virus (ECTV), the agent of mousepox, as a model of viral infection, our group has shown that mice increasingly succumb to infection as they age since they lack fully mature NK cells that are vital for protection. We have previously shown that under homeostatic conditions, aged B6 mice have a deficit in the maturation of NK cells in the blood, spleen, lymph nodes and bone marrow and that this results in impaired migration of NK cells to the draining lymph node and susceptibility to mousepox. Using an adoptive transfer model, we demonstrate that the age of the host and not the donor bone marrow cells affects the recovery of mature NK cells in the bone marrow suggesting that the radio resistant mesenchymal/stromal cells in aged cannot support the full maturation of NK cells whereas the hematopoietic precursor cells of aged are capable of generating mature NK cells. Aged mice also display

reduced proliferation of NK cells, multiple signs of immaturity and altered expression of activating and inhibitory receptors on NK cells. Gene array and q-PCR analysis of bone marrow stromal cells reveal that the message for Type I collagens, Col1a1 and Col1a2 is dramatically reduced in the stromal subset of bone marrow of aged mice. At the protein level, we found that receptors for Type I collagen, CD49b integrin which is a late stage marker of NK cell differentiation, is also significantly down-regulated on total NK cells and mature NK cell subsets in the bone marrow of aged mice when compared with young mice. Moreover, we show that treatment with complexes of the cytokine IL-15 and IL-15R α induced massive expansion of immature but not of mature NK cells in aged mice. As a result, IL-15/IL-15 α treatment did not restore resistance to mousepox in aged mice. Because many infectious diseases in humans spread systemically via lymph nodes and NK cell-deficient patients have significantly increased incidence of viral infections, it is possible that a defective NK cell maturation and migration also contributes to the increased susceptibility of older people to at least some viruses. Our mouse model provides a unique avenue to investigate NK cell dysfunctions with strong potential to advance our knowledge on causes of increased susceptibility to viral disease that we believe should be translatable to humans.

P2251

Board Number: B880

Functional and Phenotypic Alterations in NK Cells in Amnesic Mild Cognitive Impairment (aMCI) and Mild Alzheimer Patients.

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Alzheimer's disease (AD) is a progressive irreversible neurological brain disorder characterized by accumulation of β -amyloidogenic peptides, amyloid plaques and neurofibrillar tangles. Inflammation and immune alterations have been linked to AD, suggesting that the peripheral immune system plays a role during the asymptomatic period of AD. NK cells are part of innate immunosurveillance against intracellular pathogens and malignancy, their role in AD remains controversial. We have investigated changes in NK cell phenotypes and functions in amnesic mild cognitive impairment (aMCI) (n = 10) and mild Alzheimer (mAD) patients (n = 11) and, healthy elderly controls (n = 10). Patients selected according to NINCDS-ADRDA criteria were classified using neuropsychological assessment tests. Phenotype analysis revealed differences in expression of CD16 (increased in mAD), NKG2A (decreased in aMCI), TLR2 and TLR9 (both decreased in mAD). Percentage of IL-18 receptor β was lower in aMCI without differences in IL-12 receptors β 1- and β 2 for the three groups. Functional assays revealed that NK cell killing activity and degranulation (CD107 expression) were unchanged in the three groups. In contrast, expression of CD95 receptor was increased in aMCI and mAD. Granzyme B expression and cytokine production (TNF α , IFN γ) were increased in aMCI not in mAD. CCL19- but not CCL21-dependent chemotaxis was decreased in aMCI and mAD, despite the fact that CCR7 expression was increased in

aMCI. Our data suggest that a limited number of alterations that are predominantly observed in peripheral NK cells of mAD patients may affect more severely their capacity to respond to pathological aggression than aMCI subjects in progressive development of AD.

P2252

Board Number: B881

NK-mediated immune surveillance of senescent cells.

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NK-cell-mediated immune surveillance of senescent cells is one component of the coordinated process whereby cellular senescence limits the extent of liver fibrosis and facilitates wound repair. Recent studies also suggest that senescent cell recognition and clearance by immune cells promotes tumor regression in established tumors. Our results demonstrate that senescent cells are preferentially recognized and killed by NK cells. We show that DNA damage-induced senescent fibroblasts specifically upregulate the NKG2D immune ligands MICA, ULBP1, ULBP2 and ULBP5. Furthermore, we demonstrate that this immune ligand upregulation is mediated by ERK signaling and Rb, independent of p53 and NFκB. Importantly, the upregulation of MICA and ULBP2 on the cell surface of senescent cells is pivotal for the NK-mediated recognition, since interference with the receptor-ligands interactions inhibits the NK-mediated clearance both *in vitro* and *in vivo* to limit liver fibrosis and facilitate tissue repair. Lastly, we demonstrate that the granule exocytosis pathway, but not the death receptor pathway, is necessary for the specific killing of senescent fibroblasts and stellate cells by NK cells and participates in the clearance of senescent activated HSCs to limit liver fibrosis. We suggest that this pathway bias is mediated by the upregulation of Dcr2, a decoy receptor for the death ligand TRAIL, by senescent cells. Therefore, NK-cell-mediated recognition of senescent cells via NKG2D ligands and killing through NKG2D signaling and the granule exocytosis pathway contributes to immune surveillance of senescent cells *in vitro* and *in vivo*.

P2253

Board Number: B882

Comparing Nutrient Restrictions and its Gender Specific Effects on Metabolic Output in *Drosophila melanogaster*.

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Aging is a complex process that has a variety of underlying causes. Reactive oxygen species (ROS) cause oxidative damage to proteins, lipids and carbohydrates, and contribute to aging. Although calorie restriction has been linked to an increased lifespan, previous results showed that *Drosophila melanogaster* raised on a high nutrient diet lived longer than those on a nutrient restricted diet. As

expected, adult males raised on the high nutrient food had higher triglyceride levels but females raised on the high nutrient food had lower triglyceride levels compared to those raised on the nutrient restricted diet. ROS levels were detected by measuring SOD (superoxide dismutase) expression and were found not to vary between the two nutrient conditions in males. However in females, no expression of cytosolic SOD (SOD1) was detected but mitochondrial SOD (SOD2) expression was robust. Both SOD1 and SOD2 enzyme activity require divalent cations and the nutrient restricted food contains higher levels of iron. Studying iron and its direct or indirect effect on other major and trace minerals as the cause of these unfamiliar results opens windows of opportunity to fully understand iron-response elements (IREs) along the 5' UTR. These minerals, which contribute to everyday movement, could be the answer to metabolic mutations that occur under specific nutrient conditions, and contribute to slow aging.

P2254

Board Number: B883

D-galactose decreases mitoNEET (CISD1) levels in HepG2 cells.

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The monosaccharide D-galactose has been used as a tool to study accelerated aging, oxidative stress, and tissue damage in animal models. Supraphysiological levels of D-galactose accelerate aging in rodents by decreasing motor activity and superoxide dismutase activity, and by increasing production of reactive oxygen species (ROS). However, the underlying mechanism of galactose action is still poorly resolved. MitoNEET was discovered as target protein of the drug pioglitazone, a compound widely used to increase peripheral insulin sensitivity. MitoNEET is a small iron-sulfur cluster [2Fe-2S] containing protein localized to mitochondrial membranes. MitoNEET deficiency decreases iron content in the mitochondrial matrix and increases production of ROS in both animal and plant cells. Pioglitazone ameliorates galactose mediated memory deficit in a mouse model of aging. To investigate the role of mitoNEET in galactose-induced aging-related changes, we exposed human hepatocellular carcinoma cells (HepG2) to 10 mM D-galactose and measured endogenous mitoNEET levels by immunoblotting. Galactose treatment for 2 weeks significantly reduced mitoNEET levels in HepG2 cells. This effect was observed both in presence or absence of glucose in the culture medium. D-galactose also decreased levels of mitoNEET-GFP (green fluorescent protein) under the CMV promoter in stably transfected HepG2 cells. Since transcription of the endogenous mitoNEET and mitoNEET--GFP is regulated by different promoters, D-galactose likely increases degradation of mitoNEET. Furthermore, addition of 15 μ M pioglitazone in presence of galactose significantly increased the levels of mitoNEET while concentrations above 30 μ M, significantly reduced mitoNEET levels as compared to galactose treatment alone. This is the first report that links mitoNEET levels to the D-galactose induced model of accelerated aging processes. [This work was funded by an Eastern Illinois University Council on Faculty Research (CFR) grant to M.A.M and a Research and Creativity Award to S.P.]

P2255**Board Number: B884****Mitochondria fusion and fission mutants and their roles in cellular degeneration.**V.M. Khare¹, P. Tran^{1,2};¹Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA, ²CNRS UMR144, Institut Curie, Paris, France

Mitochondria are highly dynamic organelles that undergo fission and fusion. Defects in their dynamics have been linked to diverse diseases. However, at the single-cell level, it is less clear how defects in fission and fusion modify cellular physiology and influence cell health. To gain new insights into the connection between mitochondria dynamics and cell health, we examined four *S. pombe* genes which are conserved and regulate mitochondria dynamics: *dnm1+* and *fis1+* (fission), and *msp1+* and *ugo1+* (fusion). Wild type fission yeast cells have long mitochondria that align with the cell long-axis. Deletion of either *dnm1* (*dnm1Δ*) or *fis1* (*fis1Δ*) resulted in hyper-fused mitochondria. Conversely, deletion of *msp1* (*msp1Δ*) or *ugo1* (*ugo1Δ*) resulted in fragmented mitochondria. All individual mutants showed reduced cell doubling time compared to wild type, especially *msp1Δ* which had a cell cycle time 3-fold longer than wild type. Interestingly, in a population under good nutrient condition, these mutants exhibited very low cell death not different than wild type. We propose that, at least in one cell cycle, the mitochondria fragmented mutant *msp1Δ* extends the chronological lifespan of the cell. We are currently testing this hypothesis by examining cellular processes such as cell polarity and growth, and cell division.

P2256**Board Number: B885****Involvement of hexosamine biosynthetic metabolites in endoplasmic reticulum stress and aging.**P. Kim¹, A. Estrada², M. Hudson², J. Kirkwood³, J. Prenni³, D. Wang², Y. Wei², M. Pagliassotti²;¹Biological Sciences, Grambling State University, Grambling, LA, ²Food Science and Human Nutrition, Colorado State University, Fort Collins, CO, ³Proteomics and Metabolomics Facility, Colorado State University, Fort Collins, CO

Endoplasmic reticulum (ER) stress and the collapse of proteostasis are recognized as important events in both disease and aging. Glycosylation is one mechanism used by the ER to promote proteostasis. The hexosamine biosynthetic pathway (HBP) provides substrate, in the form of UDP-N-acetylglucosamine, for both N- and O-linked glycosylation. Recent evidence has demonstrated that enhanced HBP activity not only confers resistance to tunicamycin (a drug that inhibits protein glycosylation), but can also promote longevity in *C. elegans*. The aim of this study was to examine the HBP in the aging liver of mice and tunicamycin-induced ER stress in liver cells. Here, we report that HBP end products UDP-N-acetylhexosamines were reduced in the liver of old (27-29 mo) compared to young (4-6 mo) mice. The liver of old mice was characterized by ER stress, activation of the inflammasome, and injury. We also

report that glucosamine, an intermediate metabolite in the HBP, reduced ER stress induced by tunicamycin in H4IIE rat hepatoma cells. Using real time RT-PCR, we found that treatment with 5 µg/ml tunicamycin for 16 hours upregulated splicing of XBP1 and expression of CHOP and GADD34, whereas providing 5 mM glucosamine during the final 10 hours of tunicamycin insult significantly decreased these ER stress markers. These preliminary data suggest that the ability to maintain HBP end products is impaired in aging and that the HBP may be linked to aging-induced ER stress in the liver.

P2257

Board Number: B886

The role of Hsf1 in the maintenance of the endoplasmic reticulum homeostasis during chronological aging in *S. cerevisiae*.

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Cellular aging is a ubiquitous process whose molecular mechanisms remain largely unclear. A deeper understanding of cellular aging is important not only in the context of general lifespan, but also to understand the mechanisms behind the toxicity of aging-related diseases, like Alzheimer's and Huntington's disease. The Heat Shock Response (HSR) is a conserved cellular response to various stresses, including exposure to elevated temperatures and the presence of misfolded proteins in the cytosol. In both yeast and humans, the HSR is primarily regulated by the highly conserved heat shock transcription factor, Hsf1. Several genetic studies indicate that the HSR plays a major role in healthy cellular aging, and age-dependent impairment of the heat shock response is well documented in different organisms, including yeast, worms, and flies. Accumulation of misfolded proteins is associated with aging in various model organisms, and to resolve misfolded secretory protein burden, cells have evolved the Unfolded Protein Response (UPR) to help re-establish protein folding homeostasis in the endoplasmic reticulum. The goal of our study is to characterize the interplay between the HSR and UPR during chronological aging in yeast. Chronological aging in yeast is achieved through maintenance of cells in the stationary growth phase, where nutrients have been depleted and cells remain in a quiescent state. We found that chronologically aged yeast cells display increased UPR activation when compared to young cells, suggesting that changes in the ER misfolded protein burden occur during chronological aging. Furthermore, cells with no functional UPR (*ire1Δ*), or that are unable to upregulate the ER chaperone Kar2 (UPREd), have shorter chronological lifespan (the amount of time that stationary phase yeast cells remain viable) than their wild-type counterparts demonstrating that up-regulation of the UPR is essential for healthy chronological aging. Our work also illustrates that constitutive activity of the HSR through overexpression of HSF1 alleviates ER stress and promotes growth in *ire1Δ* cells, and that cells expressing a mutated Hsf1 exhibit decreased chronological lifespan. Ongoing studies aim to determine whether Hsf1 expression in *ire1Δ* and UPREd cells rescues their stunted aging phenotype, and to elucidate the underlying mechanisms. Taken together, our data reveal a role for the HSR in alleviating secretory protein stress and extending chronological lifespan.

P2258**Board Number: B887****Functional Analysis of Vacuolar Membrane Protein Ssg1 Which May Be Involved in the Regulation of Lifespan in *Saccharomyces cerevisiae*.**T. Koyama¹, R. Tsubakiyama¹, K. Kume¹, M. Mizunuma¹;¹Graduate School of Advanced Sciences of Matter, Hiroshima University, Higashi-Hiroshima, Japan

Aging is an unavoidable phenomenon and common to all living organisms. However, the molecular mechanism associated with aging and longevity is little understood. We previously found that a mutation of *SAH1* gene, which encodes S-adenosylhomocysteine (SAH) hydrolase, affected cell growth and chronological lifespan. In fact, the *sah1-1* mutant has a growth defect and a short lifespan. Moreover, we have isolated the long-lived mutant by screening for the suppression of the growth defect of the *sah1-1* mutant. This dominant-mutation was named *SSG1* (Spontaneous Suppressor of Growth-delay of the *sah1-1*). Interestingly, the *SSG1* single mutant was also shown to have a longer lifespan than a wild-type yeast. Therefore, the Ssg1 protein is predicted to be a key factor in longevity. To investigate the function of the Ssg1 protein, we constructed EGFP-Ssg1 and observed its localization by using a fluorescence microscope. We found that the Ssg1 protein was strongly localized in the vacuolar membrane during exponential growth phase and disappeared in stationary phase. Next, to examine whether it was functionally significant for the Ssg1 protein to localize in the vacuolar membrane, we constructed a double mutant between *SSG1* and *vps33* which has a defect in vacuole formation. It was revealed that the Ssg1 protein was spread entire in the cell and the longevity effect of the *SSG1* mutation was canceled by the deletion of *VPS33*, even though the Ssg1 protein was produced at roughly the same level as the *SSG1* single mutant. This suggests the importance of functions in the vacuolar membrane of the Ssg1 protein. Interestingly, we found that the *SSG1* mutation highly accumulated S-adenosylmethionine (SAM) and SAH. Considering these results, it was expected that the *SSG1* mutation promoted SAM and SAH accumulations in the vacuole and we measured the amounts of SAM and SAH both in the cytoplasmic compartment and in the vacuolar compartment. Accordingly, it turned out that SAM and SAH accumulated in the vacuole as a result of the *SSG1* mutation. In conclusion, it is suggested that the Ssg1 protein localized in the vacuolar membrane, and may work as a transporter to carry SAM and/or SAH into the vacuole. We are investigating the role of the Ssg1 in the regulation of longevity in detail.

P2259**Board Number: B888****SCZ genes, which suppress Ca²⁺-sensitivity of *zds1Δ*, are implicated in the regulation of life span in the budding yeast *Saccharomyces cerevisiae*.**I. Yamana¹, R. Tsubakiyama¹, K. Kume¹, M. Mizunuma¹;¹Graduate School of Advanced Sciences of Matter, Hiroshima University, Higashi-Hiroshima, Japan

In any organism, aging is characterized as the gradual decline of multitude of physiological functions which present major risk factors for most diseases and finally leads to death. Much of our understanding of aging derives from studies in model organisms, such as yeasts, worms and mice. However, to date we have not understood of what actually causes aging.

In eukaryotic cells, the calcium ion (Ca²⁺) is involved in the regulation of various cellular functions as a signal molecule. The *zds1Δ* strain of *Saccharomyces cerevisiae* shows sensitivity to Ca²⁺ and the Ca²⁺-induced phenotypes which are G2 cell cycle arrest, and polarized bud growth, due to the activation of cellular Ca²⁺-signaling pathways mediated by calcineurin (CN) and the Mpk1 MAP kinase pathway. To uncover the growth control regulated by the the Ca²⁺-signaling pathways in more detail, we screened for suppressor mutants of the Ca²⁺-sensitive phenotypes of *zds1Δ* cells (*scz* mutants). We have clarified into 14 complementation groups and *SCZ14* was found to be allelic to *SIR3*, which is involved in the regulation of gene expression and lifespan. Therefore, it is expected that other mutants would also regulate lifespan. So we have conducted a lifespan analysis using all identified *scz* mutants.

In budding yeast, two different paradigms of aging have been developed: replicative lifespan (RLS) and chronological lifespan (CLS). Previously, we measured the RLS (the number of daughter cells produced by a mother cell) of all *scz* single mutants. All mutants turned out to have a reduction of their RLS, suggesting that SCZ genes are implicated in the regulation of aging. In particular, the increase in CN activity by the *zds1* deletion reduced RLS. Further, increasing the extracellular Ca²⁺ level causes a shortened RLS in WT. Therefore, it was suggested that Ca²⁺ homeostasis influences RLS.

To investigate whether the SCZ genes affect CLS, we measured the CLS (the length of time that a mother cell can survive in a non-dividing, quiescence-like state) of *scz1~14* single and *zds1Δ scz1~14* double mutants. In contrast to RLS, some mutants extended CLS. One of the long-lived *scz* mutants was the *cnb1Δ* strain, which encodes the regulatory subunit of CN. It has been reported that CN-deficient animals have an extended lifespan through the induction of autophagy in *C. elegans*. We examined whether autophagy is affected by the deletion of CN. However, *cnb1Δ* did not induce autophagy in yeast, indicating that *cnb1Δ* extends lifespan in a different manner. We are currently investigating how the CN affects lifespan.

P2260**Board Number: B889****Nicotinamide reverses senescent cell phenotype: possible differential role of phosphatidylinositol 3-kinase subtype enzymes.**K. Matuoka¹, K. Sasaki¹, K. Nakanishi¹, T. Iida¹, K.Y. Chen²;¹Chiba Institute of Science, Chiba-ken, Japan, ²Rutgers, State University of New Jersey, Piscataway, NJ

Phosphatidylinositol 3-kinase (PI3K) is an enzyme family functioning in energy metabolism and intracellular signaling pathways for cell survival, proliferation and aging. When cells age, they express a set of senescence-specific phenotypes, including enlarged cell shape, decrease in proliferation and saturation density and expression of senescence-associated β -galactosidase (SA-gal). We have observed that nicotinamide (NAM), an NAD⁺ precursor, or PI3K inhibitors can reduce certain aging cell phenotypes. We wonder what could be the causes that lead to this phenomenon. With the recognition of the age-dependent decrease in cellular motility, we wonder whether this decrease may be related to other senescence-specific events. We found that treatment of senescent human fibroblastic cells with NAM (4-10 mM) for 10-20 h led to changes in cell morphology and motility, which were then followed by initiation of cell division and a decrease in SA-gal. Similar effects were also observed with PI3K inhibitors, albeit with different potency: BEZ235 was the most effective one, followed by ZSTK474, LY294002 and then wortmannin. As both NAM and PI3K are known to be involved in energy metabolism, we also examined the effect of sirtuin- and AMP kinase-modulating chemicals, including resveratrol, splitomicin, AICAR and dorsomorphin, on senescence-specific phenotypes. None of these chemicals could fully reproduce the effect of NAM on aging, showing that the action of NAM in reversing the senescent phenotypes may be related to the PI3K system, but not to pathways involving sirtuin or AMP kinase. On the other hand, we also observed that the phosphorylation of Akt, the key signaling molecule downstream of PI3K, was reduced in senescent cells, but tended to be higher after the exposure to NAM. This may imply Akt not playing a direct target of the PI3K inhibitors or NAM. As the PI3K system comprises several classes and subtypes, it is possible that the differential effect of PI3K inhibitors on senescent cells could be related to the different spectrum of their actions on PI3K subtype enzyme species. In this regard we found that class I β mRNA was reduced in senescent cells, whereas the class II α and class II β mRNA levels in senescent cells were higher. We speculate that these different PI3K species may have different roles in regulating age-dependent biological events. They may also have different responses to various known PI3K inhibitors. Current work is aiming at using these inhibitors to elucidate the role of individual PI3K subtypes in aging process.

P2261**Board Number: B890****Cockayne syndrome protein B (CSB) localizes to the nucleolus to regulate rDNA transcription in response to oxidative stress.**E.L. Boetefuer¹, H. Fan²;¹Department of Biology, University of Pennsylvania, Philadelphia, PA, ²Department of Genetics, University of Pennsylvania, Philadelphia, PA

The object of this study is to determine the role of Cockayne syndrome protein B (CSB) in protecting cells against oxidative stress. Cockayne syndrome is a severe, autosomal recessive disorder characterized by sun sensitivity, neurological and developmental defects, and progeroid features. CSB is an ATP-dependent chromatin remodeler and is critical for relieving oxidative stress; however, little is known as to how CSB accomplishes its specific functions in this process. Here, we perform a complementation test, which demonstrates the presence of CSB promotes cell survival upon oxidative stress. Furthermore, chromatin remodeling is required for CSB's protective role. Indirect immunofluorescence was used to examine the cellular localization of CSB, which shows oxidative stress also results in the accumulation of CSB within the nucleolus, the site of rDNA transcription and processing. Mutation analysis indicates this localization requires both the ATPase domain and C-terminus of CSB. RT-qPCR analysis of nascent rDNA transcription indicates CSB functions to suppress rDNA transcription upon oxidative stress. Taken together, we demonstrate a role of CSB in protecting cells during oxidative stress by downregulating nascent rDNA transcription within the nucleolus.

P2262**Board Number: B891****Changes in gene expression profile in human mesenchymal stem cells young and senescent treated with titanium microparticles.**J.C. Tavares¹, D.A. Cornelio², G.O. Brasil³, I.T. Job e Meira², S.R. Batistuzzo de Medeiros²;¹FACISA/UFRN, Santa Cruz, Brazil, ²DBG, UFRN, Natal, Brazil, ³DBG, UFRN, Natl, Brazil

Human mesenchymal stem cells (hMSC) are multipotent cells and have been used in cell therapy. Replicative senescence or acquisition of tumorigenic features can appear after long time cultivation in vitro. Considering the interference of passages and titanium in the gene expression, we decided to evaluate the transcriptome of hMSC isolated from umbilical cord vein. The cells were cultivated until early passages (young cells) and until late passages (senescent cells) without and with exposition to titanium microparticles at 24-hour and 144-hour time point (50 e 100 microparticles/cell, respectively). The cords were obtained from three donors, two with normal karyotype, identified as hMSC/n, and one with an inversion chromosome (46,XY,inv(3)(p13-p25)), called hMSC/inv. Using microarray analysis, we demonstrated a range of differentially expressed genes (DEG) between the young hMSC/inv and young hMSC/n. Interestingly, there were more DEG when they become senescent. There was no DEG between

hMSC/n untreated and treated with titanium for all doses and time tested during the treatment. When highest titanium dose at 24-hour, we verified more DGE between hMSC/inv young and senescent. Only in senescent hMSC/inv treated with titanium at 144-hour there was DEG in a titanium dose-dependent manner. The data clearly show the presence of possible new biomarkers of cellular senescence in both hMSC untreated and treated: DIO2, IGFBP5, MRV1, SCUBE3, KRTAP1-5, HAS3, KRT34, GALNT5, FOXE1, KRT19, PLCB4. We also determine new networks for DEG when comparing senescent and young cells, with the bottlenecks MMP1, THBS1 (senescent hMSC/n) and EGF (senescent hMSC/inv). In senescent hMSC/inv, others functional annotations of segregation of chromosomes, mitotic spindle formation and mitosis and proliferation of tumor lines were the most represented within of functional categories. Genes inserted into functional annotations related to tumors were more pronounced in our analysis, some of them associated to giant cell tumor of bone. The functional categories over-represented in senescent cells without titanium particles were related to cellular development, cell growth and proliferation, cell death, cell signaling and interaction and cell movement. In young cells, the functional category most enriched at 50 particles/cell and at 100 particles/cell dose at 24-hour was cellular development. Specifically, when using 100 particles/cell, other functional categories were represented such as: cell cycle, cellular movement, cell death and survival, cell growth and proliferation and cell morphology. In senescent cells, in this same condition of treatment we identified genes classified as functional category of DNA replication, recombination and repair. Taken together, these data is relevant to improve the knowledge concerning molecular mechanism of the cellular senescence, in presence or absence of titanium particle treatment. Lastly, our data reveal particular genes involved in the initial stages of mesenchymal carcinogenesis. Financial support CNPq

P2263

Board Number: B892

Sirt6 deficiency produces intrinsic osteoblast defects leading to low-turnover (age-related) osteoporosis.

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Several pathological conditions resembling human aging, including osteopenia, have been observed in sirtuin 6 (Sirt6)-deficient mice. However, the mechanism by which Sirt6 controls bone metabolism is unknown. The original report ascribing osteopenia to decreased circulating IGF1 is likely incorrect. Here, we show that loss of Sirt6 function produces a low-turnover osteoporosis caused by impaired bone formation by osteoblasts and bone resorption by osteoclasts. Micro CT analysis of long bones from Sirt6-deficient mice revealed decreased bone volume, trabecular bone mineral density, trabecular number, trabecular thickness, and cortical thickness compared to wild-type mice. Bone histomorphometry revealed impaired bone formation and bone resorption along with decreased osteoblast and osteoclast numbers. Osteoblastogenesis and osteoclastogenesis, which is an osteoblast free system, were impaired in culture. Furthermore, mineralization by Sirt6-null osteoblasts was remarkably suppressed in vitro.

Mechanistically, we discovered that Sirt6 interacts with Sp7, one of the essential transcription factors for osteoblastogenesis, and deacetylates histone H3 lysine 9 (H3K9) at the Dickkopf-1 (Dkk1) promoter in wild-type osteoblasts. In fact, Dkk1 was not detected at protein levels in wild-type osteoblasts with BMP-2 treatment. In contrast, Dkk1 protein expression was detected in Sirt6-null osteoblasts under the same conditions. Given that Dkk1 is a potent Wnt antagonist and negatively regulates osteoblastogenesis and mineralization, up-regulated Dkk1 protein expression might impact osteoblastic development and function in Sirt6-deficient mice. We also found enhanced production of osteoprotegerin (OPG), an inhibitor of osteoclastogenesis, in Sirt6-null osteoblasts with BMP-2 treatment, while OPG production is suppressed by BMP-2 in wild-type cells. Therefore, enhanced OPG expression in osteoblasts may be the cause of impaired osteoclast development and function in Sirt6-deficient mice. In fact, the numbers of osteoclasts were attenuated in co-culture assays using primary Sirt6-null osteoblasts and Sirt6-null bone marrow mononuclear cells compared to the co-culture assay using the both wild-type cells. Since Sirt6-deficient mice reveal characteristic features of age-related osteoporosis in humans, the cellular and molecular mechanisms of Sirt6 in bone turnover are potential therapeutic targets for age-related bone loss.

P2264

Board Number: B893

Starvation and inhibition of lysosomal function increased tau secretion by primary cortical neurons.

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Recent studies have demonstrated that human tau can be secreted by neurons and non-neuronal cells, an event linked to the propagation of tau pathology in the brain. In the present study, we confirmed that under physiological conditions, one tau-positive band was detected in the culture medium with an anti-tau antibody recognizing total tau and the Tau-1 antibody directed against unphosphorylated tau. We then examined whether tau secretion was modified upon insults. Tau secretion was increased by starvation [Earle's Balanced Salt Solution (EBSS)], inhibition of lysosomal function (leupeptin) and when both of these conditions were superimposed, this combined treatment having the most important effects on tau secretion. Interestingly, the pattern of tau secretion was distinct from that of control neurons when neurons were treated either with EBSS alone or EBSS + leupeptin. In these conditions, three tau-positive bands were detected in the culture medium. Two of these three bands were immunoreactive to Tau-1 antibody revealing that at least two tau species were released upon these treatments. Collectively, our results indicate that insults such as nutrient deprivation and lysosomal dysfunction observed in neurodegenerative diseases could result in an increase of tau secretion and propagation of tau pathology in the brain.

P2265**Board Number: B894****Earlier onset of mitophagy contributes to the prolonged chronological lifespan of *ras2Δ* yeast strain.**L. Belicova¹, A. Pichova¹;¹Institute of Microbiology, The Academy of Sciences of the Czech Republic, Prague, Czech Republic

Ras/adenylate cyclase (AC)/PKA pathway is one of the major pathways regulating chronological aging in various model organisms. Deletion of *RAS2* gene in yeast *Saccharomyces cerevisiae* attenuates this pathway and results in prolonged chronological lifespan of a *ras2Δ* strain. Mitochondria play an important role in cell aging and their quality control involves autophagic removal of mitochondria – mitophagy. We aimed at investigating whether and how mitochondria and their quality control process – mitophagy – contribute to the prolonged lifespan of the *ras2Δ* strain. Viability, oxygen consumption, reactive oxygen species (ROS) production, and mitochondrial network morphology were assessed in wild-type and *ras2Δ* strain. To evaluate the importance of mitophagy, a key mitophagy gene *ATG11* was deleted in respective strains (thus creating *atg11Δ* and double knock-out *ras2Δ atg11Δ*). The results showed that the extended lifespan of the *ras2Δ* strain is most probably enhanced by earlier removal of damaged mitochondria by mitophagy and by reducing their respiratory metabolism. In our study, a disruption of mitophagy resulted in higher ROS production and loss of viability in the *ras2Δ* strain. Inability to perform mitophagy did not influence the wild-type strain in any of the studied parameters. The strain *atg11Δ* therefore compensates the *ATG11* deletion by alternative processes. Our results point to a possible interconnection between mitophagy and Ras/AC/PKA signaling pathway. Understanding the interplay of mitophagy and Ras pathway might help to elucidate how cells ensure the mitochondria quality control in the aging process.

Autophagy**P2266****Board Number: B895****TRIM family proteins regulate selective autophagy and act as a new class of autophagic receptors.**M. Mandell¹, T. Kimura¹, V. Deretic¹;¹Molecular Genetics and Microbiology, University of New Mexico, Albuquerque, NM

Tripartite-motif containing proteins (TRIMs) constitute a large family with effects on cell cycle control, innate immunity, inflammation, and antiviral defense. We screened the TRIM family for roles in autophagy and found that more than half of TRIMs modulated autophagy. For most TRIMs tested to date (TRIM5α, TRIM6, TRIM17, TRIM22, and TRIM49), this is accomplished by TRIMs serving as

platforms for the assembly of autophagy initiation factors ULK1 and Beclin 1 in their activated states, although TRIMs such as TRIM55 also employ additional and/or alternative mechanisms to promote autophagy induction. We next considered whether TRIMs played additional roles as selective autophagy receptors, this being of interest due to a present dearth of the known selective autophagy receptors in mammalian cells. All TRIMs tested interacted with mammalian Atg8s (mAtg8s), a class of proteins functionally associated with autophagosome membranes. To find a putative autophagic cargo for a TRIM, we used TRIM5 α , as it has a well-known physiologically relevant target: retroviral capsid. We found that knock-down of autophagy factors in rhesus cells increased retroviral infection with a virus (HIV) known to be restricted by rhesus TRIM5 α but had no effect on a retrovirus (SIV) not recognized by rhesus TRIM5 α . We furthermore found that autophagic degradation of retroviral capsid by TRIM5 α required TRIM5 α 's binding to mAtg8s, thus establishing TRIM5 α as an autophagic receptor. Whereas the target of TRIM5 α is an exogenous protein (retroviral capsid), we have identified several endogenous proteins that interact with and are delivered for autophagic degradation by specific TRIMs. Thus, the function of TRIMs as autophagic receptor—regulators is conserved among the family. Based on our findings and the sheer number of TRIMs (>70 genes in the human genome), we propose that TRIMs direct and conduct selective autophagy of diverse substrates in metazoan cells.

P2267

Board Number: B896

REGULATION OF AGO2 FUNCTION AND DEGRADATION DURING GLUTATHIONE DEPLETION.

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miRNAs interact with Argonaute (Ago) proteins to form RNA-induced silencing complex (RISC) and guide them to specific target sites located in the 3'-UTR of target mRNAs leading to translational repression and deadenylation-induced mRNA degradation. Autophagy is the major intracellular degradation system by which cytoplasmic proteins and organelles are delivered and degraded in the lysosome. Ago2 is directed for degradation as miRNA-free entities by the selective autophagy receptor NDP52. We have previously described that a glutathione (GSH) deficiency triggers autophagy. The aim of this work was to evaluate the degradation of Ago2 during a glutathione depletion condition. Here, we show that HeLa cells treated with BSO, a potent inhibitor of glutathione biosynthesis, decreases the protein level of Ago2. However, when HeLa cells were treated with BSO and chloroquine to inhibit autophagy, accumulation of Ago2 was observed. We found that Ago2 localization and its binding site to the 5'-end of the miRNA are important for its degradation during this condition. Finally GSH depletion and autophagy inhibition affect the interaction of Ago2 with Let7a miRNA. These results suggest that GSH and autophagy machinery are important for the normal gene silencing through miRNAs.

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P2268

Board Number: B897

The Small GTPase Rab7 as a Central Regulator of Hepatocellular Lipophagy.

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Autophagy is a central mechanism by which hepatocytes catabolize lipid droplets (LDs). Currently, the regulatory mechanisms that control this important process are poorly defined. The small GTPase Rab7 has been implicated in the late endocytic pathway and is known to associate with LDs, although its role in LD breakdown has not been tested. In this study, we demonstrate that Rab7 is indispensable for LD breakdown in hepatocytes subjected to nutrient deprivation. Importantly, Rab7 is dramatically activated in cells placed under nutrient stress; this activation is required for the trafficking of both multivesicular bodies (MVBs) and lysosomes to the LD surface during autophagic catabolism. Depletion of Rab7 leads to gross morphological changes of MVBs, lysosomes, and autophagosomes simultaneously reducing the physical interactions between these compartments and therefore attenuating hepatocellular lipophagy. These findings provide additional support for the role of autophagy in hepatocellular LD catabolism while implicating the small GTPase Rab7 as a key regulatory component of this essential process.

P2269

Board Number: B898

Selective VPS34 inhibitor blocks autophagy and uncovers a role for NCOA4 in ferritin degradation and iron homeostasis in vivo.

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Cells rely on autophagy to clear misfolded proteins and damaged organelles to maintain cellular homeostasis. In this study we use a novel VPS34 inhibitor to screen for new autophagy substrates. The inhibitor acutely blocks autophagy, de novo lipidation of LC3 and leads to the stabilization of autophagy substrates. By performing ubiquitin-affinity proteomics on VPS34 inhibited cells we identified new autophagy substrates including NCOA4 which accumulates in ATG7 deficient cells and co-localizes with autolysosomes. NCOA4 directly binds ferritin heavy chain-1 (FTH1) to target the ~450 kDa iron-binding ferritin complex to autolysosomes following starvation or iron depletion. Interestingly, Ncoa4^{-/-} mice display a profound accumulation of iron in splenic macrophages, which are critical for the reutilization of iron from engulfed red blood cells. Taken together this study provides a novel mechanism for selective autophagy of ferritin and reveals a previously unappreciated role for autophagy and NCOA4 in the control of iron homeostasis in vivo.

P2270**Board Number: B899****Ubiquitin-dependent ULK1 negative feedback regulation constraints autophagy activity.**C. Liu^{1,2}, L. Pang^{1,2}, P. Wu¹, M. Lin¹, R. Chen^{1,2,3};¹Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan, ²Institute of Biochemical Sciences, College of Life Science, National Taiwan University, Taipei, Taiwan, ³Institute of Molecular Medicine, College of Medicine, National Taiwan University, Taipei, Taiwan

Autophagy, a cellular self-eating mechanism, is important for maintaining cell survival and tissue homeostasis in response to various stressed conditions, such as starvation. Although the mechanism of autophagy induction has been well studied, it remains elusive how cells modulate the level of autophagy once it has been induced. KLHL20 is a substrate adaptor of Cullin3-family ubiquitin ligase. Here, we show that ULK1, a serine/threonine kinase critical for autophagy initiation, is a substrate of the Cul3-KLHL20 ubiquitin E3 ligase complex. KLHL20-mediated ubiquitination leads to ULK1 proteasomal degradation. In responses to autophagy induction, the activation and autophosphorylation of ULK1 enhances its affinity to KLHL20, which results in an increased ULK1 ubiquitination and degradation. We further show that this KLHL20-dependent ULK1 regulation controls the amplitude and duration of autophagy to prevent prolonged or overly activated autophagy. This study identifies a novel regulatory mechanism for ULK1 and suggests a role of this regulation in autophagy termination.

P2271**Board Number: B900****A Kinase Independent Role for EGF Receptor in Autophagy Initiation.**X. Tan¹, R.A. Anderson¹;¹Molecular Pharmacology, University of Wisconsin-Madison, Madison, WI

Autophagy is a highly conserved self-eating pathway for homeostasis maintenance in all eukaryotic cells. Misregulation of autophagy is linked to human diseases including cancers. It has been generally established that autophagy prevents tumor initiation in normal cells, but in established tumors autophagy may promote tumor progression by facilitating tumor cell survival in metabolically stressed conditions. The Epidermal Growth Factor Receptor (EGFR) is an oncogenic receptor tyrosine kinase over-expressed and/or over-activated in numerous human cancers. EGFR activation suppresses autophagy in tumor cells, and inhibition of EGFR signaling by tyrosine kinase inhibitors (TKIs) is associated with enhanced autophagy in many cancer cell lines. However, the mechanism by which EGFR inactivation upregulates autophagy is not clear.

We have shown that EGFR inactivation resulting from either serum starvation or EGFR TKI treatment induces EGFR accumulation at late endosomes. The endosomal oncoprotein LPTM4B directly and

selectively interacts with inactive EGFR and is required for the accumulation of EGFR at late endosomes upon serum starvation. Most significantly, both LAPTM4B and EGFR are required for autophagy initiation, and re-expression of either wild type or kinase dead EGFR rescues autophagy in EGFR knockdown cells. LAPTM4B and inactive EGFR interact with the Sec5 exocyst subcomplex that is also required for autophagy. Together, LAPTM4B, inactive EGFR and Sec5 collaborate to control the disassociation of Rubicon (an autophagy inhibitor) from the Beclin 1 complex to initiate autophagosome formation. These findings reveal a kinase independent role for EGFR in autophagy initiation upon serum starvation or EGFR TKI treatment. This could be a mechanism for the prosurvival role of EGFR in established tumors and for the EGFR TKI resistance in clinical therapies.

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P2272

Board Number: B901

Detecting the Function of the Small GTP-Binding Protein Arl1 in Autophagy.

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Autophagy is a cellular degradation process that sequesters components such as organelles or proteins into a double-membrane structure called the autophagosome, which then fuses with the lysosome or vacuole for the hydrolysis of those components. Autophagy can be triggered in response to a variety of intracellular or environmental stresses such as pathogen invasion, starvation, or unfolded-protein stress. Once induced, autophagy is performed and regulated by several Atg proteins. Factors that control membrane traffic are also essential for each step of autophagy. Here we demonstrate that a small GTP-binding protein named Arl1, which belongs to the Arf/Arl/Sar protein family is involved in starvation-induced autophagy in *Saccharomyces cerevisiae*, but only under high temperature stress. Arl1 normally controls the retrograde trafficking from the endosome to the trans-Golgi and is important for proteins to be targeted to the vacuole. Using an assay which measures the degradation of GFP-Atg8 to free GFP to monitor bulk autophagy under different temperatures, we found that starved cells lacking the ARL1 gene show defects in bulk autophagy at elevated temperature, 37°C but not at 30°C. The same conclusion was reached with assays using fluorescence microscopy to monitor the GFP-Atg8 signal in the cells under different conditions as well as the Pho8Δ60 activity assay to quantify the magnitude of autophagy. Furthermore, experiments with different nucleotide binding alleles of Arl1 demonstrated that the GTP restrictive form of Arl1 (Arl1[Q72L]) can rescue the defect. Finally, we found that loss of Ypt6, another GTP binding protein that is synthetically lethal with Arl1, shows similar defects in autophagy under heat stress. Future work will focus on determining the mechanism for the roles of both Arl1 and Ypt6 in autophagy.

P2273**Board Number: B902****Regulation of Autophagy by ULK3, an Atg1-family kinase.**C.R. Braden¹, T.P. Neufeld¹;¹GCD, University of Minnesota, Minneapolis, MN

Macroautophagy (hereafter autophagy) is a key cellular housekeeping process in which organelles and macromolecules are packaged in a double walled membrane, the autophagosome, and delivered to the lysosome via membrane fusion. Aggregated and long-lived soluble proteins and malfunctioning organelles are all subject to destruction via autophagy, meaning its proper regulation is a requirement for successful maintenance of cellular function. As an aggregate clearance mechanism, autophagy is of interest in aggregate-prone disease states such as Alzheimer's. As a requirement for clearing damaged mitochondria, it is a key element in the study of Parkinson's. As a pro-survival pathway in a variety of stress conditions including starvation, autophagy is scrutinized in the development of cancer treatments, and it is a target of inhibition in chemotherapy.

While many signaling pathways lead to autophagy, most require the involvement of serine-threonine kinase Atg1 (*Drosophila melanogaster*) or its mammalian homologues Unc-51-like kinases 1 & 2 (ULK1 & 2). Under beneficial growth conditions, Atg1 is subject to inhibitory phosphorylation by the Target of Rapamycin (TOR). When inhibition is relieved, such as during amino-acid starvation, Atg1 induces autophagy in a manner dependent on its kinase domain, its binding partner Atg13, and a variety of complex members and downstream targets.

Recently, stimuli that can induce autophagy independent of Atg1/ULK1/2 have been described, and it is unclear how these signals interact with the core autophagy machinery. The ULK family includes an additional member, ULK3, whose potential role in autophagy is poorly characterized, and we hypothesize that ULK3 may represent this missing link. Here we demonstrate that like Atg1, ULK3 is capable of inducing autophagy in the *Drosophila* fat body. Induction of autophagy by Ulk3 overexpression does not require Atg1, but does require the Atg1 binding partner Atg13. Sequence analysis of ULK3 shows a pair of MIT domains, reminiscent of the MIT domain structures identified in Atg1 required for Atg13 interaction. However, unlike Atg1, ULK3 is not required for starvation-induced autophagy. Interestingly, Ulk3 mutation does delay onset of autophagy during treatment with the proteasome inhibitor MG132, a relative of the chemotherapy drug bortezomib. Ulk3 mutant animals are viable, but demonstrate reduced survival rates compared to wild type animals. Taken together, our data suggest ULK3 may act as a junction point between the canonical autophagy signaling cascade and a novel upstream pathway, possibly connected to proteasome malfunction.

P2274**Board Number: B903****The LRRK2 inhibitor GSK2578215A activates autophagy mediated by mitochondrial fission and mitochondrial-derived ROS.**S. Saez-Atienzar^{1,2,3}, L. Bonet-Ponce¹, J. blesa¹, F. Romero¹, J. Jordan³, M. Galindo²;¹Dpt Physiology, Universidad Catolica de Valencia, Valencia, Spain, ²Unidad de Neuropsicofarmacología Traslacional, Complejo Hospitalario Universitario de Albacete, Albacete, Spain, ³Grupo de Neurofarmacología, Universidad de Castilla-La Mancha, Albacete, Spain

Parkinson's disease (PD) constitutes the main motor disorder and the second neurodegenerative disease after Alzheimer's disease. Etiology of PD remains unknown, but both environmental and genetic factors have been implicated. Among the genes associated with PD is the leucine-rich repeat kinase 2 (LRRK2). Mutations in LRRK2 have been described in familial Parkinson's disease and sporadic disease. However, the role of LRRK2 is still unknown and its inhibition is a promising pharmacological target in PD treatment. We used the LRRK2 inhibitor 2 arylmethoxy-5-substituent-N-arylbenzamide (GSK257815A) at a concentration of 1 nM to study the effects of LRRK2 inhibition on the well-established dopaminergic cell line SH-SY5Y. We focused our study on crucial neurodegenerative features such as mitochondrial dynamics, autophagy and cell death. We found out that LRRK2 inhibition, either by pharmacological inhibition (using GSK2578215A and LRRK2-In-1) or molecularly by siRNA to knockdown LRRK2, activates autophagy since it increased the number of autophagosomes after 9 hours of treatment. Using the lysosome inhibitor chloroquine (CQ) and the mRFP-GFP-LC3 plasmid, we denoted that this increment results from both an increase in autophagosomes synthesis and an impairment of its degradation. LRRK2 inhibition also induced mitochondrial fragmentation mediated by Drp-1, in a time-dependent manner, starting at 6 hours after treatment. The use of MDIVI-1, a mitochondrial division inhibitor, avoided autophagy. This data strongly suggest that mitochondrial fragmentation is indeed an early step preceding autophagy. In order to ascertain the role of autophagy, we used autophagy disruptors that revealed the participation of autophagy as a cytoprotective response by removing damaged mitochondria by mitophagy. In order to know the role of mitochondrial-derived ROS, we analyzed 4-hydroxy-2-nonenal (4-HNE). We observe accumulation of 4-HNE after GSK2578215A treatment. The mitochondrial-targeted reactive oxygen species scavenger MitoQ positioned these species as second messengers between mitochondrial morphological alterations and autophagy. In summary, our data show how LRRK2 inhibition activates a cellular response where autophagy results in cytoprotection downstream of a disruption of the mitochondrial dynamics balance via a Drp1-dependent process. Our study suggests that drugs which modulate the functions of Drp-1 or mitochondrial-ROS may represent new opportunities to slow down neurodegeneration linked to Parkinson's disease.

P2275**Board Number: B904****Optineurin is an autophagy receptor for damaged mitochondria in parkin-mediated mitophagy that is disrupted by an ALS-linked mutation.**Y.C. Wong¹, E.L. Holzbaur²;¹Physiology, University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA, ²Department of Physiology, University of Pennsylvania, Perelman School of Medicine, Philadelphia, PA

Mitophagy is a cellular quality control pathway in which the E3 ubiquitin ligase parkin targets damaged mitochondria for degradation by autophagosomes. We examined the role of optineurin in mitophagy, as mutations in optineurin are causative for glaucoma and the neurodegenerative disease Amyotrophic lateral sclerosis (ALS), diseases in which mitochondrial dysfunction has been implicated. Using live cell imaging, we demonstrate the parkin-dependent recruitment of optineurin to mitochondria damaged by depolarization or reactive oxygen species (ROS). Parkin's E3 ubiquitin ligase activity is required to ubiquitinate outer mitochondrial membrane proteins, allowing optineurin to stably associate with ubiquitinated mitochondria via its ubiquitin binding domain (UBD); in the absence of parkin, optineurin transiently localizes to damaged mitochondrial tips. Following optineurin recruitment, the omegasome protein DFCP1 (double FYVE-containing protein 1) is transiently recruited to damaged mitochondria to initialize autophagosome formation. Optineurin then recruits autophagosome formation around damaged mitochondria via its LC3 interaction region (LIR) domain. Depletion of endogenous optineurin inhibits LC3 recruitment to mitochondria, leading to increased mtDNA content within cells. This defect in autophagosome formation and mitochondrial degradation is rescued by wildtype optineurin, but not by an ALS-associated UBAN mutant (E478G), or by a LIR mutation in optineurin. Optineurin and the autophagy receptor p62/SQSTM1 are independently recruited to different domains on damaged mitochondria, and p62/SQSTM1 knockdown does not affect either optineurin or LC3-recruitment to damaged mitochondria. Thus, our results demonstrates a critical role for optineurin as an autophagy receptor in parkin-mediated mitophagy. Importantly, as mutations in parkin are linked to Parkinson's disease, our study further indicates that defects in a single pathway lead to neurodegenerative diseases with distinct pathologies.

P2276**Board Number: B905****L84 interferes with autophagic flux and contributes to Parkinsonism.**K. Lee¹, S. Namkoong¹, I. Jang², R. Park¹, J. Park¹;¹Division of Biological science and technology, Yonsei University, Wonju, Korea, ²kbsi, Daejeon, Korea

L84 is an antihypertensive drug that has been used to control the high blood pressure, and one of its adverse effects is that long term treatment of L84 often causes Parkinson's disease in human and animal. However, the precise mechanism of L84 to induce Parkinson's disease is not clear. Here we report that one possible mechanism of L84 to induce Parkinson's disease. L84 treatment induced the

autophagosome formation by inhibiting the autophagic flux, and also accumulates p62, an autophagy adapter molecule.

P2277

Board Number: B906

Autophagy as a cytoprotective response to ethanol-induced damage in human Retinal Pigment Epithelial cells.

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Retinal Pigment Epithelium (RPE) plays a crucial role in the physiology and pathophysiology of the retina due to its location and metabolism. Oxidative damage has been demonstrated as a pathogenic mechanism in several retinal diseases, and reactive oxygen species are certainly important by-products of ethanol (EtOH) metabolism. Under EtOH exposure, autophagy has been shown to exert a protective effect in different cellular and animal models.

Thus we challenged a widely accepted model of human RPE cells (ARPE-19 cells) with 80, 200, 400 and 600 mM EtOH. 24 h after EtOH addition, we found out that EtOH treatment increased GFP-LC3 puncta, starting at 80 mM. LC3-II conversion by western blot was also detected after EtOH treatment, in a concentration dependent-manner. Interestingly, using both the mRFP-GFP-LC3 plasmid and the lysosome inhibitor chloroquine, we observed an increase in autophagy flux, in a concentration-dependent manner. In fact, our data pointed out an implication of the mTOR pathway. Moreover, autophagy activation seemed to be Beclin1 and ATG5-ATG12 dependent, as well. Mitochondrial morphology seemed to be clearly altered under EtOH exposure, leading to an apparent increase in mitochondrial fission. Interestingly, mitochondrial fission appeared to be dynamin-related protein 1 (Drp1) and optic atrophy 1 (OPA1) dependent but Bax independent. Consequently we noticed that mitochondrial autophagy is increased, in a concentration-dependent manner. In fact, we clearly demonstrated that mitochondrial ROS stimulates autophagy. An increase in 2',7'-dichlorofluorescein fluorescence and accumulation of lipid peroxidation products, such as 4-hydroxy-nonenal (4-HNE), among others, were confirmed. The characterization of these structures confirmed their nature as aggresomes. Furthermore, we observed that autophagy efficiently degrades 4-HNE aggresomes. Finally, after autophagy inhibition, we noticed an over 2 fold increase in cell death, only in EtOH-treated cells.

Hence, our data strongly suggest that autophagy plays a cytoprotective role in ARPE-19 cells under EtOH damage, by degrading fragmented mitochondria and 4-HNE aggresomes. Herein, we describe the central implication of autophagy in human RPE cells upon oxidative stress induced by EtOH, with possible implications for other conditions and diseases.

P2278**Board Number: B907****DUAL ROLE OF TFEB IN MICROGLIA.**N. Pastore¹, T. Huynh¹, A. Ballabio²;¹Jan and Dan Duncan Neurological Research Institute, Texas Children Hospital, Houston, TX, ²Telethon Institute of Genetics and Medicine, Naples, Italy

Transcription factor EB (TFEB) belongs to the MITF/TFE subfamily of basic helix–loop–helix leucine-zipper (bHLH-LZ) transcription factors. In mammalian cells, TFEB is known to exert a transcriptional control of autophagy and lysosomal biogenesis. Recently, it has been reported that TFEB is also important for host defense against infections. Lysosomal activity is essential to autophagy, a cellular pathway that delivers cytoplasmic components to lysosomes for degradation and is involved in many diseases. Autophagy has been extensively implicated in immunity. Mutations in autophagy genes increase susceptibility to infections by intracellular pathogens. Microglia plays a crucial role in maintaining brain tissue homeostasis. In pathological conditions, microglia releases pro-inflammatory cytokines and cytotoxic factors, which aggravate the progression of neurodegenerative diseases. In this study we investigated the role of TFEB in microglia in vitro, in BV2 microglial cells, and in vivo, in LysM-Cre; TFEB flox/flox and LysM-Cre; TFEB-3xflag mice that have reduced or enhanced expression of TFEB specifically in macrophages. Real time PCR analysis demonstrated that knockdown of TFEB in microglial cells results in increased levels of pro-inflammatory cytokines (IL1 β , IL6, TNF α) in basal conditions, while the inflammation is reduced after LPS challenge. Brains from LysM-Cre; TFEB flox/flox mice show increased levels of pro-inflammatory cytokines and inflammation, as demonstrated by NF κ B and JNK levels. Serum IL1 β levels are also increased in these mice. LPS-treated LysM-Cre; TFEB flox/flox mice show a decrease in inflammatory response compared to control mice. Overexpression of TFEB either in cells or in mice results in a different inflammatory response depending on the levels of TFEB: low levels overexpression result in increased response to LPS treatment, while high levels overexpression result in reduced inflammation after LPS challenge. Our data suggest that TFEB has a dual role in the microglia depending on cell activation status.

P2279**Board Number: B908****Bax inhibitor 1 Enhances Autophagy in Cyclosporine A Treatment and Reduces Renal Dysfunction.**R.K. Yadav¹, B. Li¹, G. Lee¹, H. Kim², H. Chae^{3,4};¹Department of pharmacology, Chonbuk National University, Jeonju, Korea, Dem People's Rep, ²School of Dentistry, Wonkwang University, Iksan, Iksan, Korea, Dem People's Rep, ³Department of Pharmacology, Chonbuk National University medical school, Jeonju, Korea, ⁴Department of Pharmacology, Chonbuk National University medical school, Jeonju, Korea

Cyclosporine A (CsA), a calcineurin inhibitor, improves allograft survival in solid organ transplantation. Previous evidence indicates that CsA induces autophagy, and that chronic treatment with CsA results in accumulation of autophagosomes and reduced autophagic clearance. Autophagy is a bulk protein degradation system that likely plays an important role in normal proximal tubule function and recovery from acute kidney injury. In the present study, we used human kidney tubular cells to demonstrate that CsA-induced autophagy is highly stimulated in Bax inhibitor-1 (BI-1)-overexpressing cells (BI-1 cells). BI-1 cells exhibit special activity involving highly maintained lysosome activation that promotes autophagosome and lysosome fusion. In BI-1 cells treated with CsA, transcription and nuclear translocation of TFEB is increased. In addition, treatment of BI-1 knockout mice with CsA resulted in decreased autophagosome and lysosome formation. We also found that BI-1 knockout mice were susceptible to CsA-induced kidney injury. Taken together, BI-1 appears to serve as a protective mechanism against CsA-induced nephrotoxicity through autophagy both in vitro and in vivo.

P2280**Board Number: B909****The autophagosome marker LC3 undergoes regulated targeting to the nucleus and nucleolus.**L.J. Kraft^{1,2}, J. Dowler¹, A.K. Kenworthy¹;¹Mol Physiol Biophys, Vanderbilt University Medical Center, Nashville, TN, ²Harvard University, Boston, MA

Autophagy is a lysosomal degradation pathway that is important for the maintenance of cellular homeostasis. Although the formation of autophagosomes occurs in the cytoplasm, it is increasingly evident that several key proteins in the autophagy pathway shuttle in and out of the nucleus. We previously reported that LC3, a protein that participates in autophagosome formation and cargo selection, is enriched in the nucleus in a slowly diffusing form. However, the mechanisms that retain LC3 in the nucleus and control its nuclear dynamics remain poorly understood. To address this, we used a combination of fluorescence microscopy and FRAP to study Venus-LC3 in HeLa cells. We find that mutating residues involved in binding of LC3 to other proteins and RNA moderately decreases its

nucleocytoplasmic ratio, but disrupting LC3 lipid modification does not change its nucleocytoplasmic ratio. This suggests that the majority of LC3 in the nucleus is soluble, and that it is retained in the nucleus by interactions with either proteins or RNA. Consistent with this, soluble nuclear LC3 diffuses more slowly than predicted for a monomer. Perturbing the autophagy pathway with rapamycin or chloroquine has little effect on LC3's nuclear localization, but does change the apparent size of LC3-associated complexes. This implies that soluble nuclear LC3 associates with complexes with functions possibly related to autophagy. Unexpectedly, we also find that nuclear LC3 is enriched within the nucleolus. We show that the triple arginine motif of LC3, a region previously shown to bind to both proteins and RNA, shares similarities with a consensus nucleolar detention sequence. Furthermore, perturbation of LC3's triple arginine motif completely abolishes its localization to the nucleolus. Together, these findings reveal a potential role for both protein-protein and protein-RNA interactions in targeting LC3 to the nucleus and nucleolus.

P2281

Board Number: B910

ATG5 and ATG7 fulfill essential and non-redundant roles in human hematopoietic stem/progenitor cells.

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Autophagy is a homeostatic mechanism that is crucial in the degradation of damaged organelles and misfolded proteins. This catabolic process is characterized by the formation of a double membrane vesicle called autophagosome that after engulfing cellular components will fuse with a lysosome for degradation of the cargo. Autophagy can be triggered in response to stress conditions such as starvation, energy limitation, hypoxia and DNA damage. Surprisingly, the role of autophagy in the regulation human hematopoietic stem cell (HSC) function remains unclear. Besides their ability to differentiate into all blood cell lineages, HSCs have the capacity to self renew and remain quiescence for the lifelong maintenance of hematopoiesis. Thus, HSCs require stringent housekeeping mechanisms safeguarding their integrity and protecting them from accumulation of damage. Currently little is known concerning the regulation of autophagy in hematopoietic stem and progenitor cells, or how this may change during myeloid differentiation.

Autophagy was analyzed by Western blotting and FACS. In addition colony forming cell (CFC) assays, long term culture-initiating cell (LTC-IC) assays, apoptosis measurements, cell-cycle analysis and reactive oxygen species (ROS) measurements were performed.

We demonstrated that autophagy is functional in human hematopoietic stem/progenitor cells (HSPCs). Robust accumulation of the autophagy markers LC3-II and p62 were observed in HSPCs treated with bafilomycin-A1 (BafA1) or hydroxychloroquine (HCQ). In addition, CD34⁺CD38⁻ stem cell-enriched cells showed enhanced accumulation of cyto-ID (a marker for autophagic vesicles) compared to CD34⁺CD38⁺ progenitor cells upon BafA1 and HCQ treatment. To study the functional consequences of autophagy,

ATG5 or ATG7 were knockdown in CD34⁺ cells, using lentiviral shRNAs. Downregulation of ATG5 and ATG7 resulted in a marked decrease in progenitor frequencies, which coincided with a strong reduction in expansion of CD34⁺ cells. In addition, a 3-fold reduction in stem cell frequencies was observed. The reduction in HSPCs was not due to impaired differentiation, but was at least in part due to reduced cell cycle progression and an increase in apoptosis. The changes in cell cycle and increased apoptosis coincided with increased expression of p53 and downstream proapoptotic target genes BAX, PUMA and PHLDA3 and cell cycle inhibitor p21. Additionally, ROS levels were increased after ATG5 and ATG7 knockdown. The increased apoptosis in shATG5 and shATG7 transduced cells might be triggered by elevated ROS levels.

Taken together, our data demonstrate that autophagy is an important survival mechanism for human HSCs and their progeny.

P2282

Board Number: B911

Autophagy protects against neurodegeneration in a *Drosophila* model of C9orf72-mediated ALS.

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A recently identified GGGGCC hexanucleotide repeat expansion (HRE) mutation in C9orf72 is the most common genetic cause of amyotrophic lateral sclerosis (ALS). In a forward genetic modifier screen of HRE toxicity in *Drosophila*, we discovered that the loss of function of p62 strongly suppresses HRE-induced neurodegeneration. p62 plays a critical role in autophagy, a self-degradation process that prevents the accumulation of toxic misfolded proteins in many neurodegenerative disorders. p62 binds and delivers ubiquitinated proteins to the autophagosome, eventually leading to lysosomal degradation of both p62 and its cargos. Defects in this process cause accumulation of p62 as well as its cargos. Interestingly, we find that flies expressing the HRE display increased p62 protein levels, intracellular p62 protein aggregates in motor neurons, and impaired synaptic transmission at the neuromuscular junction, consistent with the findings reported in C9orf72 ALS patient autopsies. Furthermore, we find that genetic and/or pharmacological activation of the autophagic pathway significantly suppresses neurodegeneration concomitantly with p62 aggregation, strongly suggesting that autophagic clearance of p62-positive aggregates can rescue C9orf72-related neurotoxicity. Hence, we propose that C9orf72 HRE causes cytotoxic protein aggregations containing p62, which can be rescued by increasing autophagy. Our study suggests that activation of autophagy may be a therapeutic strategy for C9orf72-mediated ALS.

P2283**Board Number: B912****BNIP3 Induces Full-length PINK1 Accumulation to Promote Mitophagy.**T. Zhang¹, L. Xue², L. Li¹, C. Tang¹, Z. Wan¹, R. Wang¹, Z. Zeng¹, W. Andy Tao², Z. Zhang³;¹ Central South University, State Key Lab of Medical Genetics, Changsha, China, ²Purdue University, Department of Biochemistry, West Lafayette, IN, ³ Central South University, State Key Laboratory of Medical Genetics, Changsha, China

Mutations in *PINK1* cause early onset familial Parkinson's disease (PD). PINK1 is accumulated on followed by recruiting parkin to the outer membrane of damaged mitochondria to promote mitophagy. Here, we demonstrate that BNIP3, a mitochondrial BH3-only protein, interacts with PINK1 to promote stabilization and accumulation of full length PINK1 on outer membrane of damaged mitochondria, therefore, to facilitate parkin recruitment to mitochondria. The results suggest that BNIP3 plays an essential role in PINK1/parkin-mediated mitophagy and likely contributes to PD pathogenesis.

Keywords: Parkinson's disease, PINK1, BNIP3, mitophagy

Systems and Synthetic Cell Biology**P2284****Board Number: B914****Measuring fast gene dynamics in single cells with timelapse luminescence microscopy.**A. Mazo-Vargas¹, H. Park¹, M. Aydin¹, N. Buchler¹;¹Biology, Duke University, Durham, NC

Timelapse fluorescence microscopy is an important tool for measuring *in vivo* gene dynamics in single cells. However, fluorescent proteins are limited by slow chromophore maturation times and the cellular auto-fluorescence or photo-toxicity that arise from light excitation. An alternative is luciferase, an enzyme that emits photons and is active upon folding. However, the photon flux per luciferase is significantly lower than fluorescent proteins. To date, timelapse luminescence microscopy has been successfully used to track gene dynamics in larger organisms and for slower processes, where more total photons can be collected in one exposure. Here, we tested green, yellow, and red beetle luciferases and optimized substrate conditions for *in vivo* luminescence. By combining timelapse luminescence microscopy with a microfluidic device, we tracked the dynamics of cell cycle genes in single yeast with sub-minute time resolution over many generations. Our protocol is significantly faster and in cells with much smaller volumes than previous work. Fluorescence of an optimized reporter (Venus) lagged luminescence by 20 minutes, which is consistent with its known rate of chromophore maturation in yeast. Our work shows that luciferases are better than fluorescent proteins at faithfully tracking the underlying gene expression.

P2285**Board Number: B915****Two sequential decisions commit human cells to start the cell cycle.**S.D. Cappell¹, S.L. Spencer¹, M. Chung¹, A. Jaimovich¹, T. Meyer¹;¹Department of Chemical and Systems Biology, Stanford University, Stanford, CA

One of the most fundamental decision processes in biology is the commitment of cells to enter the cell cycle and generate two daughter cells. Cell-cycle commitment in mammalian cells is thought to be regulated by a single decision point in G1 called the Restriction Point, after which growth factors are no longer required for the cell cycle to proceed. Using live-cell microscopy of cell-cycle sensors and single-cell analysis, we have identified the moment at which cells cross the Restriction Point and find that more than three hours pass between the Restriction Point and the actual start of DNA replication.

Furthermore, we find that cell stress after the Restriction Point but before the start of DNA replication results in cell-cycle exit and a return to a pre-Restriction Point state, providing evidence for another later decision to commit to the cell cycle. We present evidence for a second bistable switch regulating S-phase entry. The trigger for this second switch is the CDK2/Cyclin E-mediated partial inactivation of APCCdh1, an E3-ligase that controls the degradation of proteins necessary for S-phase progression. We show that the switch is driven by a double negative feedback between the regulatory protein Emi1 and APCCdh1. Markedly, Emi1 functions as both an inhibitor and a substrate of the APC, which generates a bistable and irreversible switch regulating S-phase entry. Thus, cells commit to the cell cycle by making two sequential decisions. They first monitor mitogen signals to decide whether to cross the Restriction Point, and then monitor stress signals to decide whether to irreversibly inactivate APCCdh1, start DNA replication, and fully commit to the cell cycle.

P2286**Board Number: B916****Multiplexed transcriptional analysis of p53 targets in single cells.**J.R. Porter¹, B.E. Fisher¹, E. Batchelor¹;¹Laboratory of Pathology, NCI/NIH, Bethesda, MD

The transcription factor p53 is activated in response to a wide range of biological stresses. To cope with different stresses, p53 regulates the expression of over a hundred different target genes in a stress-specific manner. Understanding how stresses are relayed by p53 into distinct regulation of such a large number of genes is a major challenge. The problem is confounded by the fact that common transcriptomic methods often rely on measurements of populations of cells, which can obscure information regarding single-cell heterogeneity and the coregulation of specific targets. To address these concerns, we used a microfluidic platform to analyze the expression of nearly one hundred well-characterized p53 target genes simultaneously in individual cells. We focused on p53's dynamic response to DNA double strand breaks, which we had previously characterized as an undamped pulsatile

dynamical response. We identified distinct classes of downstream target dynamics, including oscillatory and delayed target gene expression. We also characterized target genes based on expression noise as a measure of single-cell heterogeneity. From our co-expression measurements, we identified gene regulatory sub-networks operating downstream of p53. Our findings provide new mechanistic insights into how p53 coordinates stress-specific responses in individual cells, and may provide novel therapeutic strategies for altering cell fates.

P2287

Board Number: B917

Constant Growth Rate Can Be Supported by Decreasing Energy Flux and Increasing Aerobic Glycolysis.

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Fermenting glucose in the presence of enough oxygen to support respiration, known as aerobic glycolysis, is believed to maximize growth rate. We observed increasing aerobic glycolysis during exponential growth, suggesting additional physiological roles for aerobic glycolysis. We investigated such roles in yeast batch cultures by quantifying O₂ consumption, CO₂ production, amino acids, mRNAs, proteins, posttranslational modifications, and stress sensitivity in the course of nine doublings at constant rate. During this course, the cells support a constant biomass-production rate with decreasing rates of respiration and ATP production but also decrease their stress resistance. As the respiration rate decreases, so do the levels of enzymes catalyzing rate-determining reactions of the tricarboxylic-acid cycle (providing NADH for respiration) and of mitochondrial folate-mediated NADPH production (required for oxidative defense). The findings demonstrate that exponential growth can represent not a single metabolic/physiological state but a continuum of changing states and that aerobic glycolysis can reduce the energy demands associated with respiratory metabolism and stress survival.

[http://www.cell.com/cell-reports/abstract/S2211-1247\(14\)00254-X](http://www.cell.com/cell-reports/abstract/S2211-1247(14)00254-X)

P2288

Board Number: B918

Variable stoichiometry among core ribosomal proteins.

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Understanding the regulation and structure of the eukaryotic ribosome is essential to understanding protein synthesis and its deregulation in disease. While ribosomes are believed to have a fixed

stoichiometry among their core ribosomal proteins (RPs), some experiments suggest a more variable composition. Reconciling these views requires direct and precise quantification of RPs. We used mass-spectrometry to directly quantify RPs across monosomes and polysomes of budding yeast and mouse embryonic stem cells (ESC). Our data show that the stoichiometry among core RPs in wild-type yeast cells and ESC depends both on the growth conditions and on the number of ribosomes bound per mRNA. Furthermore, we find that the fitness of cells with a deleted RP-gene is inversely proportional to the enrichment of the corresponding RP in ribosomes bound to multiple mRNAs. Together, these findings support the existence of ribosomes with distinct protein composition and physiological function.

<http://biorxiv.org/content/early/2014/05/26/005553>

P2289

Board Number: B919

How do bacterial growth rates relate to evolutionary fitness landscapes for energy-efficiency?.

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We are interested in energy flows in bacteria, and how energy gets trafficked between producing ribosomal and nonribosomal proteins under different growth conditions. We describe an analytical model that leverages extensive data on experimental growth laws to infer the underlying fitness landscape in *E. coli*. This model gives insight into some of the complex nonlinear relationships between energy utilization and ribosomal and non-ribosomal production as a function of cell growth conditions. We draw inferences about what evolution has optimized in *E. coli*. Is *E. coli* optimized for growth speed or for energy efficiency? Experimental data shows that at its replication speed limit, *E. coli* produces 4 mass equivalents of non-ribosomal proteins for every mass equivalent of ribosomes. The model shows that this ratio is expected if the bacterial fitness function is the energy efficiency of fast-growing cells. We conclude that a principal evolutionary driving force for bacteria is the energy efficiency of the fastest growing cells.

P2290

Board Number: B920

Engineering a modular synthetic cyclic AMP generator.

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Adenylyl cyclases (ACs) are key amplifiers of GPCR signaling. Mammalian cells express nine distinct AC isoforms, all of which convert ATP to the second messenger cyclic AMP (cAMP). While much is known

about the structure of ACs and the signaling pathways they control, the differential function of AC isoforms and their spatio-temporal regulation remains poorly understood. Here we describe the detailed characterization of a genetically encoded synthetic adenylyl cyclase (SYNAC) that can be generalized to any AC isoform and targeted independent of the native AC. SYNAC activity can be monitored orthogonally using FRET, and is controlled by forskolin and G α s in a manner similar to native AC. We also describe a modular extension of SYNAC that can be used to modify its activity independent of endogenous G proteins.

P2291

Board Number: B921

Cell based high throughput assay to evaluate cardiovascular safety profile of newly synthesized compounds to be nominated for clinical development.

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The optimal cell based lineage for cardiac-regeneration still remains a mystery. Here we have developed a novel cell based assay to screen novel synthetic compounds for cardiovascular safety and regenerative profiles for clinical development. The human cardiomyocytes were isolated from adult ventricular and atrial appendages, characterized, and electrically profiled for functional physiological cardiac evaluations. The electrophysiology of adult human cardiomyocyte consists of 4X4 array of platinum microelectrodes and electronic measurement system based on a 1mV rms noise level amplifier and 16 channel 16bit data acquisition card. This High throughput ElectroPhysiological Assay (HEPA) cardiac chip technology was developed by Biopico Systems Inc., for characterization of the human cardiomyocytes electrophysiology functional index for cardiovascular safety and regenerative profiles. Prior to utilizing the human cardiomyocytes they were tested positive with Real time PCR, IHC and Western Blot Analysis for the following biomarkers: ckit, Actinin, ANP, Connexin 43, Desmin, KDR, Nkx2.5, GATA-binding protein 4 (GATA4) and SERCA2. The in-vitro assay procedure was established to determine cardiovascular safety and regenerative index of newly identified led molecules. In this study we tested CEP1430, a novel anti-tumor agent targeting pancreatic stem cells for cardiovascular safety; our lead molecule had no adverse cardiovascular liability. In-vivo daily administration of CEP1430 for 20 days had no cardiovascular adverse effect. From this study we were able to conclude that we have identified a lead compound for targeting pancreatic cancer stem cells that has no adverse cardiovascular effect when monitored for 20 days with once daily administration of the compound by intraperitoneal injections. This in-vitro cell based cardiac assay enabled us to determine the cardiac safety value help us determine the molecule to be a potential candidate for clinical development.

P2292**Board Number: B922****Biosynthesis of Silver Nanoparticles using *Biden frondosa* and its Tyrosinase Activity.**Q. Abbas¹, S. Seo¹;¹Department of Biology, College of Natural Sciences, Kongju National University, Kongju, Korea

Herbal Nanoparticles gain lot of attention because of their novel drug delivery system. In this study the silver nanoparticles (AgNPs) were synthesized by using aqueous extract of *Biden frondosa*. The appearance of brown color indicates the formation of *Biden frondosa*-AgNPs. The Formation and stability of AgNPs were determined by UV–Vis spectroscopy; Fourier Transform Infrared (FTIR) data confirm the presence of various aromatic and –OH moieties. The formation of herbal AgNPs further assured by energy dispersive X-ray spectroscopy (EDX) and Scanning electron microscopy (SEM) size ranging 30-50nm. The mushroom tyrosinase inhibitory activity of synthesized AgNPs was evaluated and it was found that they exhibited potent tyrosinase inhibitory activity compared to control. The IC50 value of AgNPs was found to be 9 and that of kojic acid is 15µg/mL. AgNPs of *Biden frondosa* may be considered as potential candidate for the production of medical and cosmetic products.

P2293**Board Number: B923****Bacterial Cell Adhesion to Switchable Polymer Brushes.**T. Babar¹, A. Sidorenko¹, M. Bruist²;¹Department of Chemistry and Biochemistry, University of the Sciences, Philadelphia, PA, ²Chemistry Biochemistry, University of the Sciences, Philadelphia, PA

Polymer brushes have the potential to be used for medical supplies and as new materials for industry that require a reduction or elimination of bacterial growth. We propose that the modification of solid surfaces with switchable polymer brushes allows adherence of bacteria to a hydrophobic surface or the controlled detachment of the bacteria from a hydrophilic polymer surfaces. Based on the research by Cunliffe, *et. al* (1991), we analyzed the physical adhesion properties of *Escherichia coli* and *Salmonella typhimurium*¹ when applied to switchable hybrid molecular brush (HMB) systems. HMB's are composed of a chitosan backbone with added polystyrene (PS) and polyacrylamide (PAAm) side chains. Upon optimization of production of the brushes, with respect to the polymer thickness and wettability, the bacterial response to each surface polymer was characterized using Atomic Force Microscopy (AFM). Through AFM, the initial stage of bacterial adherence, in which the bacteria recognize the surface using responding extracellular extensions, topographical images were acquired. The images show the cellular appendages, fimbria, extended from the cell's surface while adhered to polystyrene. There is an absence of fimbria when cells are on polyacrylamide. Further experiments will be conducted to observe the surface characteristics of cells when they are allowed to grow on the brushes for longer periods of times; along with comparisons of cell adherence and surface characteristics on modified HMB's.

[1] Cunliffe, D., Smart, C.A., Alexander, C., Vulfson, E.N. Bacterial Adhesion at Synthetic Surfaces. Appl. Environ. Microbiol. 1991, 65, 4995.

P2294

Board Number: B924

Reconstitution of regulated FtsZ localization by Min protein gradients in vitro.

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The controlled placement of the division apparatus plays a key role for cell development. During cell division in *Escherichia coli* the oscillatory self-assembly of the MinCDE proteins directs the assembly of the division machinery. To investigate the minimal requirements for division site localization in a controlled environment we engineered a biomimetic system by reconstituting the cell division protein FtsZ and the Min protein system in membrane clad soft-polymer compartments. This bottom-up system allows studying complex organizational principles, such as the self-assembly of Min protein concentration gradients and controlled localization of FtsZ to the middle of a cell-like. Furthermore the role of geometric boundary conditions and minimal biochemical requirements for division site localization can be addressed.

Tissue Development and Morphogenesis 3

P2295

Board Number: B926

Actomyosin network dynamics in epithelial morphogenesis.

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The lowest energy state of a simple epithelium is a hexagonal packing composed of 3-cell junctions. To generate cell geometries away from equilibrium, two types of molecular machines are required: (1) force-generating networks to remodel or stabilize cell-cell contacts and (2) polarity determinants to control the pattern of cell shape changes. Coordination of cell shape changes in a group of cells is required for proper tissue morphology and function. Dysregulated tissue morphogenesis underlies pathologies such as developmental defects and cancer.

As a model of epithelial morphogenesis, we study a dramatic alignment in the *Drosophila* embryonic epidermis that produces columns of aligned, rectangular cells. These columns give rise to lines of hooks required for larval mobility, thus the geometry of this epithelium is key to its function. Past work in our

lab has suggested that actomyosin-mediated junction remodeling events that convert 3-cell junctions to 4-cell junctions produce this rectilinear arrangement. Recently, we have characterized the cell shape changes in this epithelium at higher temporal resolution. We find that the straightening of Dorsal-Ventral (DV) interfaces between cell columns does not always temporally coordinate with junction remodeling events. By live imaging of fluorescently tagged myosin light chain, we observe two separate pools of myosin with different behaviors. First, actomyosin cables stably co-localize with the adherens junctions of straightening DV interfaces throughout alignment. Second, an apical pool of myosin located medially in aligning cells undergoes rapid cycles of contraction and disassembly. Based on these findings, we hypothesize that two distinct actomyosin networks, regulated in sequence or parallel, exert different forces on the cells in this tissue to mediate alignment: (1) actomyosin cables generate tension across all DV contacts to straighten interfaces between columns and (2) medial-apical myosin elevates force on select contacts to induce junction remodeling events. In the future, we hope to determine the polarity mechanisms that temporally regulate these two pools of actomyosin, quantitate the contractile dynamics of the medial-apical actomyosin population with respect to junction remodeling, and dissect the contribution of these distinct actomyosin pools to collective cell shape changes.

P2296

Board Number: B927

Paradoxical sleep deprivation and its consequences on the morphophysiology of ventral prostate.

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This study aimed to evaluate possible morphological and functional changes in the ventral prostate of the adults (90 days) Mongolian gerbil (*Meriones unguiculatus*) after the paradoxical sleep deprivation (also known as rapid eye movement phase – R.E.M.). The REM sleep deprivation was induced over a period of four consecutive days using the modified multiplatform method. Through this technique, the animals of treated group (PSD) remained in cages containing 18 fixed platforms, allowing their movement and access to food and water ad libitum, filled with water up to 1 cm below the surface of the platforms. When animals of the PSD group enter in the REM sleep, they lose muscle tone and fall in the water, disrupting the sleep cycle. The animals of control group (NC) remained in polypropylene cages filled with shaving woods during the whole experiment, receiving water and food ad libitum. The serum dosage for testosterone and corticosterone was performed by ELISA. Prostatic fragments were analyzed by morphometry and stereological techniques, as well as immunohistochemistry for proliferating cell nuclear antigen (PCNA) and TUNEL for apoptosis evaluation. In the prostate of PSD animals occurred an evident regression of the glandular epithelium besides some foci of hyperplasia, while the muscular stroma also becomes thinner and disorganized when compared to NC. These

morphological changes occurred without testosterone serum levels fluctuations, but an important increase in corticosterone serum levels was noted (5.37 ng / mL to 19.99 ng / ml). The animals subjected to sleep deprivation also showed a significant reduction in body weight (62.50 g to 52.75 g), however, the ventral prostate weight remained unchanged. Furthermore, there was also a decrease of PCNA-positive cells in the epithelial and stromal compartments, which was accompanied by an increase in apoptosis, particularly in the stromal region. Based on these results, it can be concluded that sleep disruption triggers important hormonal oscillations that negatively reflect in the morphophysiology of prostate gland and this condition could be considered a promoter factor of prostatic lesions.

P2297

Board Number: B928

Scarb2a gene expression is required for Notochord Development in Zebrafish.

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Scarb2 is a glycoprotein, with two transmembrane sites, several sites for N-glycosylation and a C-terminal di-leucine motif . In humans, mutations in Scarb2 were described as causing of Action Myclonus Renal Failure Syndrome (AMRF), which is diagnosed as a progressive myoclonus epilepsy. Zebrafish has three homologous genes for *scarb2* (*scarb2a*, *scarb2b* and *scarbc*). *Scarb2a* insertional-mutant was obtained in a large-scale forward genetic screening, this mutant is characterized by the presence of vesicular bodies in the brain at 1 dpf and hypopigmentation at 2 dpf both phenotypes are restored by itself at 3 dpf . Furthermore, since 1 dpf *scarb2a* mutant show a particular defect in the notochord formation, in which notochord vacuoles never reach their normal size. Whole mount in situ hybridization revealed that *scarb2a* is expressed in the brain and in the notochord at early stages. Interestingly, as development progress, *scarb2a* expression is dynamically displaced in an anterioposterior direction. We also notice, by immunostaining, that laminin is not arranged properly at the notochord basal membrane. Through electronic microscopy, we identified that specific basal membrane defects are specifically localized at the intermedial zone, however internal and external layers seems unaffected.

P2298

Board Number: B929

Dachsous, Fat and actin modulators contribute to planar cell polarity in the Drosophila ventral epidermis.

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Morphogenesis is orchestrated by the cooperation of many cells: for proper execution, cells must respond to directional information and transmit these cues to neighboring cells. To accomplish this,

epithelial cells tend to establish polarity within the plane of the epithelium, in addition to apicobasal polarity. Such planar cell polarization (PCP) allows cells to define their position within a field, thereby allowing cells to coordinately produce asymmetric responses essential for tissue function. The molecules that control planar cell polarity have been identified and well studied; polarizing systems such as the conserved Frizzled (Fz) and the Dachshous/Fat (Ds/Fat) systems allow cellular neighbors to establish and maintain polarity. Less understood is how these systems integrate with downstream effectors to choreograph asymmetric responses. Here we study the *Drosophila* ventral epidermis, an epithelium in which cells produce a stereotyped template for the production of denticles, critical for larval motility. This template is comprised of actin-based protrusions (ABPs), which are asymmetrically polarized within these cells. The Ds/Fat system is essential for the proper localization of ABPs; the current model is that Ds signals through the Fat receptor to impose polarity. We are performing functional domain analysis of Fat, in order to determine critical motifs of this molecule and to predict downstream effectors of the Ds/Fat system. In addition, through live imaging and genetic techniques, we have begun analysis of candidate cytoskeletal molecules (actin modulators, myosin II) to identify the machinery that engages in the construction and placement ABPs at the posterior edge of these cells. By working from receptor to cytoskeleton, we hope to understand how molecular polarity determinants integrate with structural effectors to functionally polarize tissue, a process critical in both development and disease.

P2299

Board Number: B930

Claudin localization to tight junctions is required for neural tube morphogenesis.

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Neurulation is a critical developmental process that converts the flat neural plate into a closed neural tube. Throughout this process the neural plate epithelium undergoes extensive remodeling including cell intercalation and apical constriction. These events require the maintenance of cell interactions within the neural epithelium through intercellular junctions. The most apical of these junctions are tight junctions (TJs), where members of the claudin family regulate apical-basal cell polarity and cell adhesion, and link the TJ to the actin cytoskeleton. We hypothesize that claudins are required for coordinating the morphogenetic events that drive neural tube closure. To test our hypothesis, we used the C-terminal domain of *Clostridium perfringens* enterotoxin (cCPE) to remove Claudin-4 and -8, and Claudin-3 and -4 from TJs of the neural and non-neural ectoderm of chick embryos respectively. Disrupting the localization of these claudins to tight junctions led to folate-resistant NTDs in 100% of embryos. In situ hybridization analysis of gene expression in the ectoderm and along anterior-posterior boundaries of the neural tube suggested that NTDs were not due to defects in ectoderm differentiation or patterning. cCPE-treated embryos displayed defects consistent with a disruption of convergent extension: a shortened anterior-posterior axis, a reduced length-to-width ratio, misshapen somites, and a broadened notochord. Transmission electron microscopy analysis of cCPE-treated embryos showed that midline

cells failed to undergo apical constriction. Furthermore, we showed reduced apical accumulation of RhoA and pMLC, an activated form of myosin II that is phosphorylated by Rho kinase (ROCK), in cCPE-treated embryos. These data indicate that claudins function upstream of RhoA/ROCK signalling to regulate actin-myosin contraction and thus, cell intercalation and apical constriction. This is the first time that claudins have been shown to directly regulate neural tube closure.

P2300

Board Number: B931

Slug/Snail2 expression during the development of squamous metaplasia in airways of patients with chronic obstructive pulmonary disease (COPD).

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Chronic obstructive pulmonary disease (COPD), a major cause of morbidity and mortality worldwide, is clinically characterized by a slow and progressive airflow obstruction and decline in lung function, resulting from a remodeling of the airways that includes several epithelium abnormalities. Inhaled agents, mainly cigarette smoke, are the main cause of COPD. The airway epithelium, first in line during exposure to inhaled environmental factors, is often injured and need to be repaired. The normal bronchial epithelium is pseudostratified, consisting of ciliated, goblet and basal cells. The latter are stem/progenitor cells that can self-renew and/or differentiate into ciliated and goblet cells in a well controlled balance to reconstitute a functional epithelium. Chronic toxic injury can repetitively perturb this balance and lead to epithelium abnormalities such as mucous cell hyperplasia or squamous metaplasia (SM). SM is frequent in the bronchial epithelium of smokers but its extent is increased in smokers with COPD compared to smokers without COPD. In COPD there is also a loss of normal pseudo stratified epithelium with transition into a fully squamous epithelium (Wilson et al, ATS2013). The objective of our work is to understand the molecular mechanisms underlying the abnormal airway epithelial repair that are specific to COPD. Slug was found to be one of the most expressed genes in mouse bronchial basal cells when compared to non-basal cells (Rock et al, 2009). We hypothesized that Slug is a key regulator of basal cell renewal and differentiation and could be involved in the development of SM. We found, by immunohistochemical labeling of bronchial biopsies, that Slug is expressed in the nuclei of cells in zones of SM in both control and COPD patients; however, lower levels are observed in biopsies from COPD. To follow the expression of Slug during the regeneration of a bronchial epithelium, we used primary human bronchial epithelial cells (HBECS) cultured in air-liquid interface (ALI). This culture system allows regeneration of a normal epithelium at high concentration of ATRA, while in conditions depleted in ATRA, SM is induced as shown by involucrin increase and cytokeratin 18 decrease of expression. Using this model, we found that stable SM is associated with a decrease of Slug protein expression. When cells are treated with TGF β , a factor previously reported to induce SM, we observed a decrease of Slug associated with SM in COPD and this correlates with a decrease in pAKT. In summary, our work shows that the more stable and mature SM found in COPD correlates with a decrease of Slug expression and that Slug may be involved in the reversibility of SM.

P2301**Board Number: B932****Male offspring of diabetic rats exhibit increased activity of MMP-2 associated to higher content of TGF- β , androgen receptor and cell proliferation in the ventral prostate.**A.A. Damasceno¹, C.P. Carvalho¹, F.A. Araujo¹, F.V. Botelho¹, R.M. Goes², D.L. Ribeiro¹;¹Histology, Institute of Biomedical Sciences/ Federal University of Uberlândia- UFU, Uberlândia, Brazil,²Biology, São Paulo State University, IBILCE/UNESP, São José Do Rio Preto, Brazil

This study presents a comprehensive view of histological and functional status in the prostate of adult rat offspring of mothers subjected to gestational diabetes. Diabetes was induced in pregnant female Wistar rats by alloxan (i.p. 100 mg/kg b.w.). The ventral prostate of male adult offspring of diabetic (DP) or normal mothers (CP) was evaluated for collagen fibres, cell death (TUNEL), immunohistochemistry (fibroblasts, smooth muscle cells, cell proliferation), western blotting (PCNA, metalloproteinases, androgen receptor-AR), TGF β -1 (ELISA), catalase and total antioxidant activity. The prostates of DP animals showed lower weight compared to the CP group and these animals also exhibited hyperglycaemia and hypotestosteronaemia. Cell proliferation and AR were higher in the diabetic offspring. The DP group also showed a reduction in α -actin, which may interfere with the reproductive function of the prostate. They also had enhanced activity of MMP-2, although the absolute content of this MMP was lower in this group. This finding was associated with an increase in TGF β -1 and a decrease in collagen distribution. The prostates of DP rats also exhibited reductions in catalase and total antioxidant activity. These data indicate that rats developing in a diabetic intrauterine environment have glycemic and hormonal changes that impact on the structure and physiology of prostate in adulthood. They exhibit increased AR expression, which may be responsible for the elevated cell proliferation. There was also stromal remodelling characterized by enhanced activity of MMP-2 and collagen degradation, even with increased TGF β -1 activation. These changes associated with increased oxidative stress may interfere with tissue architecture and glandular homeostasis, favouring the development of proliferative disorders in the prostate.

P2302**Board Number: B933****Analysis of the different functional domains relevant for chicken Scratch2 function.**T.Y. Nakamura Kanno¹, M. Soares Fogo¹, F. Monteleone Vieceli¹, C. Yan¹;¹Department of Cell and Developmental Biology, Universidade de São Paulo, São Paulo, Brazil

cScratch2 (cScrt2) is an evolutionarily conserved transcription factor that belongs to the Snail/Slug superfamily. This superfamily is involved in different developmental processes including neural differentiation, cell division, cell survival and left–right identity. As a norm, members of this family act through transcriptional repression. The repressor activity of these transcription factors depends both on the amino-terminal SNAG and carboxi-terminal Zinc-Finger domains. cScrt2 is expressed in the chick embryonic neural tube. Specifically, it is found in early postmitotic neural progenitors. Consistent with its cellular function, MYC-cScrt2 localizes to the nucleus of chick embryonic neural progenitors and HEK293T cells. An *in silico* search for subcellular localization regulatory sites revealed that residues tyrosine77 (Y77) and serine78 (S78) are possible targets for tyrosine or serine/threonine kinases. The sequence containing these residues is completely conserved between the homologues of cScrt2 in chicken, human and mice. Overexpression of cScrt2Y77F, cScrt2Y77E, cScrt2S78A and cScrt2S78D in HEK293T and chicken embryos remain in the nucleus as observed by immunofluorescence and Western Blot. The truncated protein with only the C-terminal domain is also restricted to the nucleus. Thus, it is possible that the nuclear localization of cScrt2 is mainly controlled by the Zinc-Finger domain. We thus investigated if phosphorylation of Y77 and S78 is relevant for repressor activity. All of the different Y77 and S78 point mutants decreased repressor ability as seen by luciferase assays. Given that we observe alterations in cScrt2 repressor activity independently of the charge value of the residue that substituted the endogenous Y77 or S78. We propose that all of the above mutations alter protein conformation and, as a consequence, the ability to recruit the nuclear partners involved in the cScrt2mediated transcriptional regulation. In other words, our results suggest that Y77 and S78 are relevant for the correct conformation of interaction domains involved in recruiting nuclear repressor machinery partners while the subcellular localization of cScrt2 could be controlled by the DNA-binding Zinc-Finger domain.

P2303**Board Number: B934****8-Nitro-guanosine 3',5'-cyclic monophosphate mediates elongation of the growth plate cartilage in mice.**M. Hoshino^{1,2}, Y. Miyamoto¹, K. Yoshimura¹, D. Suzuki¹, T. Akaike³, K. Mishima⁴, K. Baba², R. Kamijo¹;¹Department of Biochemistry, Showa University School of Dentistry, Tokyo, Japan, ²Department of Prosthodontics, Showa University School of Dentistry, Tokyo, Japan, ³Department of Environmental Health Sciences and Molecular Toxicology, Tohoku University Graduate School of Medicine, Miyagi, United States, ⁴Division of Pathology, Department of Oral Diagnostic Sciences, Showa University School of Dentistry, Tokyo, Japan

The majority of our bones develop through endochondral ossification, except for the collarbones and flat bones such as the skull bones and the jawbones that are formed through membranous ossification. Endochondral bone formation is regulated by growth and differentiation of chondrocytes in growth plates. It is known that nitric oxide (NO) plays important roles in controlled elongation of growth plates and hypertrophic differentiation of chondrocytes. It is also reported that C-type natriuretic peptide (CNP) stimulates proliferation and hypertrophic differentiation of chondrocytes through elevation of intracellular level of guanosine 3',5'-monophosphate (cGMP). Recently, 8-nitro-cGMP was identified in various types of cells including macrophages, glia cells, and cardiomyocytes as a novel signaling molecule. Therefore, we hypothesized that 8-nitro-cGMP may function as a mediator of proliferation and/or differentiation of chondrocytes in the growth plates. In this study, we investigated the formation and function of 8-nitro-cGMP in the growth plate cartilage in mice. Tibias excised from mice on embryonic day 16 were cultured for 4 days in the presence of CNP and either an NO synthase inhibitor, L-NAME, or its control D-NAME, the optical isomer of L-NAME. Tibias grew $1087 \pm 188.2 \mu\text{m}$ in the direction of the long axis in 4-day culture with CNP and D-NAME, whereas they grew only $766.2 \pm 181.0 \mu\text{m}$ in the culture with CNP and L-NAME. Histochemical analyses revealed that L-NAME caused suppressed elongation of the growth plate cartilage induced by CNP, indicating NO was involved in the proliferation of chondrocytes in the growth plates. Formation of 8-nitro-cGMP was detected immunohistochemically in the growth plates after cultivation with CNP, while that was inhibited by L-NAME. In addition, chemically synthesized 8-nitro-cGMP induced the *ex-vivo* growth of tibias in a concentration-dependent manner. Hence we examined the effect of chemically synthesized 8-nitro-cGMP on the proliferation and differentiation of mouse primary chondrocytes *in vitro*. 8-Nitro-cGMP induced the proliferation of chondrocytes in a concentration-dependent manner, while it did not have little effect on the expression of marker genes of hypertrophic differentiation. Collectively, it is suggested that 8-nitro-cGMP is a novel signaling molecule that facilitates proliferation of chondrocytes and elongation of growth plate cartilage.

P2304**Board Number: B935****The Role of Twist1 in endothelial cell sprouting in the lung.**T. Mammoto¹, A. Mammoto²;¹Vascular Biology Program, Boston Children's Hospital/Harvard Medical School, Boston, MA, ²Boston Children's Hosp/Harvard Med Sch, Boston, MA

Recent significant advances in stem cell research and bioengineering techniques made a great progress in utilizing biomaterials to regenerate or repair damages in simple tissues in the orthopedic and periodontal fields. However, attempts to bioengineer the structures and functions of more complex three-dimensional organs such as lungs have not been very successful. Angiogenesis –the formation of new blood capillaries- plays an important role in organ development, homeostasis, and regeneration and deregulated angiogenesis contributes to various disease states. Newly formed vasculatures not only deliver oxygen, nutrients and various cell components, but also provide instructive signals to surrounding cells in tissues. Thus, in order to engineer functional organs such as lungs, we need to understand the mechanisms that govern angiogenesis. The transcription factor, Twist1, controls mouse embryonic development and the epithelial-mesenchymal transition that occurs in lung fibrosis and lung cancer, diseases in which angiogenesis is deregulated. Here we demonstrate that Twist1 controls the expression of angiogenic factor receptor, Tie2 and endothelial cell sprouting in lung human microvascular endothelial (L-HMVE) cells. Furthermore, we recently developed a unique method to implant fibrin gel on the mouse lung, which enables the precise analysis of the biological process of host lung-derived angiogenesis and interaction between the newly formed blood vessels and other cellular (e.g., alveolar cells, immune cells) and non-cellular components in mice. When the fibrin gel supplemented with angiogenic factors vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) (0, 10 and 100 ng/ml each) is implanted on the surface of living mouse lung, host-derived CD31-positive vascular networks are constructed inside the gels 7 days after implantation in a VEGF/bFGF dose-dependent way. Importantly, angiogenesis is inhibited in fibrin gels implanted on lungs of Tie2-specific Twist1 conditional knockout mice. These results suggest that Twist1 controls angiogenesis in vitro and in the mouse lung in vivo and that Twist1 may be one of the key molecules to control lung organ morphogenesis.

P2305**Board Number: B936****Prenatal exposure to estrogen disrupts development of male and female prostate in Mongolian gerbil.**B.C. Zani¹, J.S. Maldarine², J.A. Vieira², M.M. Jesus³, F.A. Santos⁴, M.F. Biancardi⁴, B.D. Sanches³, S.R. Taboga⁵;¹Biology, UNESP, São José Do Rio Preto, Brazil, ²Biology, UNESP, S J do Rio Preto, Brazil, ³Biology, UNICAMP, Campinas, Brazil, ⁴Biological Sciences, Federal University of Goiás, Goiania, Brazil, ⁵Biology, São Paulo State University, IBILCE/UNESP, S J do Rio Preto, Brazil

Prenatal estrogenization disrupts development of prostate gland in male rodents, causing alterations in the gland growth, as well, deep changes in urethral structure. Despite much is known about the effects in male prostate, there is little research on intrauterine estrogenization in the female prostate and the differences between genders. The aim of this study was to evaluate the effects of prenatal estrogenization on the ventral prostate of Mongolian gerbils (*Meriones unguiculatus*), this species is relevant for this type of research, since the presence of functional prostate in female is almost ubiquitous. Developing prostate complexes were collected from animals at 20, 22, 24 days of postnatal life exposed to high dosages of estrogen (500 mg/kg BW/day) during perinatal life, fixed and processed for paraffin embedding and cut in series. The slides were stained with HE and immunohistochemistry essays, as well three-dimensional reconstructions were performed. Results suggest gender differences: females were more susceptible to developmental disruptions, as a decrease in the number of prostatic ducts and a increase in their thickness. Acknowledgements: FAPESP, CNPq and CAPES for financial support and scholarships.

P2306**Board Number: B937****Evaluation of Intrauterine oestrogenic exposure on female prostate development in Mongolian Gerbil.**B.D. Sanches¹, J.S. Maldarine², M.F. Biancardi³, B.C. Zani⁴, F.A. Santos³, M.M. Jesus¹, J.A. Vieira², S.R. Taboga⁵;¹Biology, UNICAMP, Campinas, Brazil, ²Biology, UNESP, S J do Rio Preto, Brazil, ³Biological Sciences, Federal University of Goiás, Goiania, Brazil, ⁴Biology, UNESP, São José Do Rio Preto, Brazil, ⁵Biology, São Paulo State University, IBILCE/UNESP, S J do Rio Preto, Brazil

Much is known about the effects of prenatal exposure to estrogen in the prostate gland in male rodents, however, there is little research on this process in the female prostate and the differences between genders. The aim of this study was to evaluate the effects of prenatal estrogenization on the ventral prostate of Mongolian gerbils (*Meriones unguiculatus*), this species is promising for this type of research, since the presence of functional prostate in female is almost ubiquitous. Developing prostate

complexes were collected from animals at 7, 14, 30 days of postnatal life exposed to high dosages of estrogen (500 mg/kg BW/day) during the prenatal life, fixed and processed for paraffin embedding and cut in series. The slides were submitted to HE staining technique and immunohistochemistry essays, as well three-dimensional reconstructions were performed. Results suggest gender differences: females were more susceptible to alterations in the urethra and prostate gland in early postnatal life, showing profound histologic changes in urethra and decreased cell proliferation in the prostate.

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P2307

Board Number: B938

A CLEM approach to studying the membrane repair process in zebrafish myofibers in vivo. Effect of high pressure freezing on fluorescence emission by fluorescent proteins.

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In order to study the process of membrane repair in myofibers in zebrafish embryos after sarcolemma injury, we designed a workflow to track the fluorescent positive myofibers (labelled with proteins of interest involved in the repair process) by LM so as to be able to eventually find them in the EM scope for high resolution investigation. For this purpose which involves the vitrification of 3-days-old embryos by HPFreezing (with a HPF device, Compact O2, Wohlwend, CH) we first tested that the fluorescence signal was preserved right after HPFreezing of our samples. We discovered that some fluorescent proteins stand the effect of the high pressure (2100 bar) and the freezing to liquid nitrogen temperature, as seen with samples visualized under liquid nitrogen with a macroscope (UV illumination), while some others do not and, as a consequence, lose their ability to emit fluorescence. Later on, after freeze substitution (FS) and plastic embedding in HM20 Lowicryl resin, the fluorescence was preserved within 250-300 nm thick sections in cases where the fluorescence was intact after the HPFreezing step, while it was extinguished in case where it was no longer detected after the vitrification step. This shows that fluorescent proteins (FPs) display a differential sensitivity to the vitrification process, probably depending on the molecular structure of FPs and/or their environment. We currently investigate which FPs are optimally suitable for CLEM studies.

P2308**Board Number: B939****Cell Rearrangement and Rosette Formation During Epithelial Morphogenesis of the *Xenopus laevis* Neural Plate.**D.S. Vijayraghavan¹, L.A. Davidson^{1,2};¹Bioengineering, University of Pittsburgh, Pittsburgh, PA, ²Developmental Biology, and Computational and Systems Biology, University of Pittsburgh, Pittsburgh, PA

During neurulation cells within the planar neural plate must coordinate their behaviors in order to deform into an elongate tubular structure. Neural tube formation is comprised of several steps including cell shape patterning of the early neural plate, neural fold formation at the lateral edge of the plate, bending at and fold convergence towards the dorsal midline, and finally fold fusion. Much of the focus of neural tube mechanics have been on the bending of the neural plate and formation of hinge points both at the midline and mediolaterally at the folds. Researchers have identified coordinated apical constriction of epithelial cells are crucial to these bending processes. However, even before bending occurs, the neural plate, as well as the whole embryo, undergoes a large convergence mediolaterally and extension in the anterior posterior direction. Convergence and extension (CE) of the neuroepithelium is thought to be driven in part by cell rearrangements through planar polarized actomyosin contractility. We have undertaken a stage-by-stage analysis of cell shape changes and local geometric patterning within the dorsal apical surface of fixed *Xenopus laevis* embryos. Cells within neural plate undergo directed cell rearrangement and form large multi-cellular rosettes where 5 or more cells share a single vertex. Directed formation and resolution of rosettes have been implicated as a driver of convergence and extension in *Drosophila* germ band epithelial morphogenesis and has been observed in later stage chick neural tube formation. Preliminary analysis of live imaging confocal data of labeled actin in the neural plate demonstrates cells frequently change neighbor both through rosette formation and more general T1 transitions. Rosettes resolution typically occurs within 1 hour after formation. High magnification time-lapses reveal a dense and highly contractile actin cortex within the neural epithelium. These observations support the notion that actively rearranging cells coincide with convergence and extension. However, it remains to be seen whether rearrangements provide the motive force driving CE or whether rearrangements merely serve to dissipate stresses acting within the epithelium.

P2309**Board Number: B940** **α integrin cytoplasmic tails can rescue the loss of Rho-family GTPase signaling in the *C. elegans* somatic gonad.**C. Meighan¹, V. Kelly¹, E. Krahe¹, A. Gaeta¹;¹Christopher Newport Univ, Newport News, VA

Integrin signaling impacts a diverse set of cell behaviors through multiple, distinct pathways. The Rho family of GTPases are integrin signaling partners that regulate cell polarity, actin dynamics, and cell migration. Though the connection between integrins and the Rho family has been studied extensively, there is still much that is unknown about the complex interplay between these protein families. To further resolve this issue, we used the nematode *C. elegans* as a simple model for integrin and Rho family function. *C. elegans* has two α integrins, *ina-1* and *pat-2*, that pair with β integrin *pat-3*. Our previous work developed chimeric α integrins which link the extracellular domain of α integrin *ina-1* to the cytoplasmic tail of α integrin *pat-2* and the extracellular domain of *pat-2* to the cytoplasmic tail of *ina-1*. These constructs showed the α integrin cytoplasmic tails have tissue specific functions during development. Here, we present evidence showing the cytoplasmic tails of α integrins *ina-1* and *pat-2* have a cell type dependent ability to bypass signaling from members of the Rho family of GTPases.

P2310**Board Number: B941****Role of shootin1 during embryonic development in zebrafish.**A. Urasaki¹, T. Matsui¹, K. Kawakami², Y. Bessho¹, N. Inagaki³;¹Bioscience, Nara Institute of Science and Technology, Nara, Japan, ²National Institute of Genetics, Mishima, Japan, ³Grad. Sch. Bio. Sci., Nara Institute of Science and Technology, Ikoma, Japan

Axon outgrowth is essential for the formation of neuronal networks. Shootin1 was identified as a protein involved in the polarization of primary cultured hippocampal neurons. Shootin1 functions as a linker “clutch” molecule that couples treadmilling F-actin and the adhesive substrate at the growth cones to promote axon outgrowth. However, little is known about the *in vivo* functions of *shootin1*. Here we identified three shootin family members (*shootin1*, *shootin2* and *shootin3*) in the zebrafish genome and analyzed the functions of *shootin1* in zebrafish. Zebrafish is widely used to study vertebrate development. Their external fertilization, rapid development and optical clarity make them well-suited for studying important cellular processes during embryonic development such as cell proliferation, migration and rearrangement. To see whether *shootin1* is expressed in zebrafish embryos, we performed whole-mount *in situ* hybridization. *shootin1* was expressed in the ventral forebrain and eyes. Next, to understand whether *shootin1* is required for forebrain and eye formation, we performed knockdown experiments. The *shootin1* knockdown embryos exhibited defects in the forebrain and eyes. Our data suggest that *shootin1* is involved in the forebrain and eye formation. Furthermore, *shootin1* was also expressed in the posterior lateral line primordia, thereby suggesting that *shootin1* may be

involved in collective cell migration of these cells. To confirm these possibilities, we are currently generating the *shootin1* mutant by using the CRISPR system in zebrafish.

P2311

Board Number: B942

RNA-binding proteins regulate dendrite morphogenesis in *C. elegans*.

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Neurons are polarized cells with complex morphology that enables processing and transmission of information to and from the neural network and surrounding environment. The growth and maintenance of neuronal processes such as dendrites are key to establishing sensory fields and the synaptic connections that control cognition, behavior, and adaptive responses to the environment. A growing body of evidence highlights mRNA transport and local translational control as key processes in generating dendritic branches, maintaining cell structure, and promoting synaptic plasticity. Therefore it is important to investigate the role of RNA-binding proteins (RBPs), which are involved in mRNA processing, transport, localization, stability, and translational control, in the regulation of dendrite form and function.

To determine the extent to which RNA-binding proteins influence dendrite morphology, Olesnicky et al. (2014) conducted a genetic screen of all RBP-encoding genes in the *Drosophila* genome and found 63 RBPs required for normal dendrite morphology in dendritic arborization (*da*) sensory neurons. An *in silico* analysis showed a high sequence-similarity among diverse species for the RBPs that affect *da* dendrite structure in *Drosophila*, including 54 homologous genes in *C. elegans*. This suggests that conserved RBPs may regulate dendrite morphology in diverse animal species. To test this hypothesis we screened these 54 RBP-encoding genes in *C. elegans* for dendrite defects using the multidendritic PVD sensory neuron as a model.

Using a combination of mutant alleles and RNA interference, our screen identified 12 conserved RBP-encoding genes that produce a reduction in the number of dendritic termini upon loss or reduction of gene function. To determine if the reduction of dendritic termini is caused by a failure to form branches, a delay in formation, or a loss of initially established branches, we performed a time-course analysis. Our results suggest that most RBPs are required for dendritic branch formation but two are required for branch maintenance and one influences the timing of branch formation. Reporter transgenes confirm that candidate genes are actively transcribed in the PVD neuron and RBP::GFP fusion proteins were used to determine the subcellular localization of each RBP. Subcellular localization and gene ontology information suggest that nuclear RBPs are involved in mRNA processing such as splicing and that cytoplasmic RBPs are likely involved in mRNA transport and translational regulation. Taken together, our results highlight a fundamental role for RBPs in dendrite morphogenesis, and suggest that these

evolutionarily conserved RBPs participate in molecular mechanisms that contribute to neurite development and maintenance in diverse animal species

P2312

Board Number: B943

Role of BMP signaling in adult tissue homeostasis.

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Background & Aim: Bone morphogenetic protein (BMP) signaling, in co-ordination with other signaling pathways control key events in organogenesis. Though BMP signaling pathway has primarily been investigated for its role during embryonic development, the identification of *Bmpr1a* and *Smad4* mutations in patients with Juvenile Polyposis Syndrome (JPS), suggests that BMP signaling may be an important regulator of adult tissue homeostasis as well. Here we report our initial observation following depleting Bmp2 and Bmp4 proteins specifically in adult mice which suggests that BMP signaling is required for adult tissue homeostasis.

Methods: We have created a conditional *Bmp2/4* double knockout mouse mutant (genotype - *Bmp2^{fl/fl}; Bmp4^{fl/fl}; ROSA26::CreER-T2*). Eight weeks old control and test mice were administered tamoxifen for seven consecutive days.

Results: Fifteen days after initiating tamoxifen injection we observed signs of mild diarrhea in these mutant mice. Molecular and histological analyses of BMP signaling depleted intestine suggests that BMP loss triggers activation of Wnt signaling in intestinal stem cell niche (crypt) resulting in remarkable increase in cell proliferation. The resultant crypt-villus morphology (ectopic, misplaced & elongated crypts with short villi), following Bmp depletion, resembles conditions similar to JPS. Loss of BMP signaling also resulted in improper nuclear positioning which is suggestive of loss of the baso-apical enterocyte polarity. Bmp ablation also causes detachment of enterocytes from the basal lamina of the villi. In our study, Bmp depletion is also associated with impaired terminal differentiation of cells from secretory lineage. This perturbation of the normal process of tissue homeostasis causes apoptosis of the cells. Interestingly, these mutant mice also exhibited hair fall following Bmp depletion. The molecular changes observed in mutant follicles is reminiscent of the changes observed in the intestine. Taken together, our results suggest that BMP signaling is an important regulator of epithelial tissue homeostasis in adult animals.

Conclusion: We strongly suggests that BMP signaling have a major regulatory role in the homeostasis of an adult epithelial tissue and a better understanding of all of this will help to utilize novel therapeutic strategies to treat conditions like intestinal polyps and cancers.

P2313**Board Number: B944****Discovery of CEP1410, a small molecule inducer that promote proliferation of pancreatic beta cells using novel micro-3D histo-culture assay procedure.**S. Satish¹, C.R. Sharma^{1,2}, J.M. Harris¹, R.R. Rodriguez¹, M.R. Sharma¹, M.B. Navel¹, N. Amezcua¹, J.R. Martinez¹, J. Jani¹, J.P. Sharma¹;¹Stem Cell Biology, Celprogen Inc, Torrance, CA, ²Celprogen Inc., Torrance, CA

Identification of novel small molecule inducers that promote proliferation of pancreatic beta-cell is an important approach to treat diabetes. This unique approach represents a potential regeneration strategy for the treatment of type 1 diabetes. Unfortunately, the lack of availability of suitable human beta cell lines makes such a discovery a challenge. Here, we adapted and successfully developed the human islets micro-3D histo-cultures for high-throughput cell based assays. This micro-3D histo-culture system of culturing human islet for high-throughput screening utilizing a thymidine analog, EdU, to detect beta-cell replication during screening. This culture system allows simultaneous monitoring of Ki67, EdU incorporation and beta cell numbers provides robust assay for beta-cell replication. Importantly, this micro-3D histo-culture system preserved the beta-cell physiological function, as measured by glucose-stimulated insulin secretion. We then performed a pilot screen of 384 compounds, observing some phenotypic effects on cells. This high-throughput human islet cell culture method can be used to assess various aspects of beta-cell biology on a relatively large number of compounds. From this screen we were able to characterize 10 lead compounds CEP1410,1412,1413,1414,1415,1416,1417,1418,1419 and 1420 that were able to increase beta cells mass and responded to glucose –stimulated insulin secretion. CEP1410, our lead compound was tested in-vivo. In diabetic rat model, once a day oral administration of CEP1410 increased the mass of Beta cells. . The gene expression profile of pancreatic Beta-cells revealed high gene expression of Insulin, glucagon, somatostatin, Glut-2 and Isl-1 on Day 20 when study was terminated.

Germ Cells, Gameogenesis, and Fertilization**P2314****Board Number: B945****ISOLATION, CHARACTERIZATION, AND GENE EXPRESSION PROFILE OF RAT EPIDIDYMAL BASAL CELLS.**M. Mandon¹, L. Hermo², D.G. Cyr¹;¹INRS-Institut Armand-Frappier, Laval, QC, ²McGill University, Montreal, QC

Epididymal basal cells are localized to the basal compartment of the epithelium. These cells have thin processes that extend along the base of the epithelium and that can also extend apically and reach the lumen of the epididymis. There is little information on the function and role of epididymal basal cells.

Various studies have suggested that these may be stem cells, be part of the immune system, regulate other epithelial cells via the production of hormones and secondary messengers, and may be regulators of the tight junctions of the blood-epididymis barrier. The objective of this study was to isolate a relatively pure fraction of epididymal basal cells and characterize their function. Immunolocalization studies indicated that integrin- $\alpha 6$ was specifically localized to basal cells in all regions of the rat epididymis. Epididymides from 42 day old rat, an age when there are no sperm in the epididymis, were enzymatically digested and cells isolated. Using a magnetic microbeads and integrin- $\alpha 6$ antibody, basal cells were isolated. Immunofluorescence using a specific marker of basal cells, cytokeratin 5 (KRT5), revealed a 90% enrichment of basal cells. Flow cytometry performed with another marker of basal cells, cyclooxygenase 1 (Cox1), confirmed this result. Electron microscopy of isolated cells also revealed morphological characteristics of basal cells. Total cellular RNA was isolated from the enriched basal cell fraction and subjected to RT-PCR using specific markers of basal cells, principle cells, dendritic cells, and clear cells. Microarray analysis indicated the basal cell fraction contained 1359 differentially expressed genes relative to other epididymal epithelial cells. Among the differentially expressed genes, numerous integrins and proto-oncogenes were expressed in the epididymis. Furthermore, the tight junction protein, claudin1, highly expressed as were connexin 43, 31.1, 30.3 and P-cadherin. Pathways analysis predicted the expression of several intracellular signaling pathways including those implicated in EGFR signaling, integrin signaling, molecular mechanisms of cancer, inflammatory response and canonical WNT signaling. These studies provide novel information on function of the epididymal basal cells and their role in the regulation of the blood-epididymis barrier. Supported by CIHR and FQRNT.

P2315

Board Number: B946

Polyglucosan Molecules Induce Testis Degeneration And Apoptosis In Germ Cells Without Affecting The Integrity And Functionality Of Sertoli Cells.

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Glycogen is the main storage form of glucose for most tissues; however, the accumulation of aberrant polyglucosan molecules can lead to degeneration and death in some cell types. Previously, we reported that the accumulation of glycogen in testis of transgenic animals overexpressing a constitutively active form of glycogen synthase (KIN-GS) enhances the apoptosis of premeiotic cells in seminiferous tubules. Similarly, the activation of endogenous glycogen synthase (GS) in a germ cell line (GC-1) stimulates the deposition of glycogen and triggers the activation of caspase 3. Here we sought to further identify the effects of glycogen storage in GC-1 and Sertoli cells (42GPA9 cell line) and the mechanism behind the pro-apoptotic activity induced. By spectrophotometric analysis, we found that glycogen synthesized in both cell lines—by expression of a superactive form of GS or by activation of endogenous GS by PTG (*Protein Targeting to Glycogen*) expression—is poorly branched. In addition, the immunodetection of

cleaved caspase 3/9 suggests that cellular death induced by polyglucosan molecules affects GC-1 but not 42GPA9 cells. The former cells showed changes in intracellular ATP and cytochrome C content after polyglucosan accumulation, thereby suggesting mitochondrial impairment and activation of an intrinsic apoptotic pathway. Furthermore, we analyzed the effects of glycogen deposition during the establishment of an in vitro blood-testis barrier. The results using a non-permeable fluorescent molecule (Evans blue) showed that, in conditions of over-synthesis of glycogen, 42GPA9 cells do not lose their capacity to generate an impermeable barrier. In the same cell line, immunodetection showed that the levels of connexin43 (Cnx43), occludin (Occl), and ZO1 proteins were not affected by glycogen accumulation. Similarly, in the KIN-GS mice, the distribution and intensity of signals for Cnx43, Occl, and ZO1 point to a Sertoli cell-only syndrome in this model, affecting the viability of male germ cells but not the viability or stability of Sertoli cells, as shown by confocal microscopy analysis. These results confirm that the accumulation of polyglucosan molecules has a selective effect—triggered by the intrinsic activation of the apoptotic pathway—in germ cells. Supported by grants: FONDECYT 3130449 (FVE), 1110508 (ICC) and 1141033 (JCS)

P2316

Board Number: B947

UNDERSTANDING THE REGULATION OF CONNEXIN 26 IN THE EPIDIDYMIS.

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Connexins (Cxs) are proteins that form gap junctions allowing neighboring cells to communicate by the diffusion of ions and small molecules (<1 kDa). Our laboratory has previously reported that Cx30.3, 31.1 and 32 are expressed in adult rat epididymis, whereas Cx26 is expressed in young animal when the epithelium is undifferentiated; suggesting a role for Cx26 in the differentiation of the epididymal epithelium. The regulation of the *Cx26* gene in the epididymis is unknown. The present objective was to elucidate the mechanisms regulating the *Cx26* gene in the epididymis through the characterization of its promoter. RLM-RACE revealed a single major transcription start site (tss) for *Cx26* at position -3829 relative to the ATG. Computational analysis revealed several AP2 and SP1 binding sites located 5' to the tss. A 1.7kb fragment of the *Cx26* promoter was amplified and cloned into a PGL3 vector containing a luciferase reporter gene. Transfection of the construct in epididymal RCE cells indicated that the promoter fragment could drive the expression of the reporter gene. Several constructs were generated by sequence deletions of the promoter and transfected into RCE cells. The luciferase assays revealed the presence of two binding response elements necessary for the expression of *Cx26*: an AP2/SP1 site and an SP1 site. The implication of those two sites was confirmed by directed mutagenesis. ChIP analysis confirmed that these factors bind to the DNA in vivo. ChIP analysis of DNA from young and pubertal animals indicated that binding of the AP2 and SP1 decreases as a function of age when *Cx26* mRNA levels decrease. DNA methylation studies were undertaken to determine whether or not changes in the methylation of the promoter might explain the decrease in AP2 and SP1 binding. The *Rhox5* promoter was used as a positive control. Results indicated that there were no changes in DNA methylation in the

promoter of young versus pubertal animals. These results indicate that the transactivation of the *Cx26* gene is regulated by AP2 and SP1 sites. The decrease in *Cx26* mRNA levels previously reported is correlated with a decrease in AP2 and SP1 binding to the promoter and this is not the result of changes in DNA methylation. Supported by NSERC.

P2317

Board Number: B948

A Point Mutation in Nonmuscle Myosin II-A Disrupts Mouse Spermatogenesis.

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Nonmuscle myosin II-A (NM II-A) is a major cytoskeletal protein that is composed of a pair of heavy chains (230 kDa, encoded by the *Myh9* gene in mice) and 2 pairs of light chains (20 kDa and 17kDa). It is ubiquitously expressed in humans and mice, and contributes to cellular processes including cell migration, cell adhesion, and cytokinesis. Humans with mutations in NM II-A have defects in platelets, and develop glomerulosclerosis, cataracts and deafness. We have generated a mouse line with a mutation frequently found in human MYH9 related disease, E1841K. In addition to modeling the above defects, male, but not female, mice homozygous for the mutation are sterile. The male mice demonstrate severe defects in sperm development. Histological analyses of homozygous testis reveal that spermatocytes arrest at the elongated spermatid stage and aberrant spermatids slough off into the lumen. Some seminiferous tubules contain vacuoles, have a large lumen and often lack germ cells, while others appear normal but completely fail to develop a lumen. Electron micrographs uncover an abnormal flattening and extension of the acrosomal vesicle, which appears to lead to the abnormal elongation of spermatids. TUNEL assays of homozygous testis show an increase in apoptotic germ cells when compared to wild type. Caudal epididymis sections have only a few malformed spermatozoa that are rarely motile. Heterozygous mice show similar, albeit less severe, defects and are fertile. Sperm counts obtained from the caudal epididymis confirm a pronounced decrease in mature sperm in homozygous mice, while the heterozygous mice show a modest decrease when compared to wild type. Collectively, these observations suggest a previously unknown, critical role for NM II-A in spermatogenesis and male fertility.

P2318**Board Number: B949****CACN-1, which interacts with the *Caenorhabditis elegans* spliceosome, is an important regulator of germline differentiation.**A.D. Cecchetelli¹, E.J. Cram¹;¹Biology, Northeastern University, Boston, MA

With its easily visible U-shaped gonad arm, *Caenorhabditis elegans* provides an excellent model to study germline stem cell differentiation and development. *C. elegans* produce sperm until the onset of adulthood, when the worm permanently switches to producing oocytes. *cacn-1*, a conserved gene originally identified as a key regulator of cell migration, also plays an important role in this sperm to oocyte switch. Depletion of *cacn-1* leads to a masculinization of the germline and/or abnormal oocytes in addition to sperm. DAPI staining revealed that *cacn-1* loss also causes endomitotically duplicating oocytes (EMO) in a significant number of gonad arms with abnormal oocytes. Previously, the lab has identified CACN-1 interacting proteins using an *in vivo* tandem-affinity purification (TAP) protocol. CACN-1 complexes were isolated and identified by mass spectrometry and characterized using bioinformatics. CACN-1 interactors grouped into a novel network suggesting that CACN-1 interacts with the *C. elegans* spliceosome. All the identified interactors were screened for germline defects using RNA interference (RNAi). Many of these interactors caused severe germline defects when depleted in wild type animals. Phenotypes included abnormal oocytes as well as a masculinization of the germline similar to *cacn-1* deficient animals. DAPI staining revealed that a majority of these proteins also cause the EMO phenotype when depleted. The results of this screen reveal that CACN-1 and interacting proteins are important in proper germline stem cell differentiation and development and that CACN-1 may be involved in pre-mRNA splicing or post-transcriptional regulation to mediate these decisions. Understanding the role of CACN-1 and its interactors in germline differentiation may lead us to a better understanding of the role of post-transcriptional regulation in stem cell differentiation.

P2319**Board Number: B1000****Identifying new players in meiosis using *C. elegans* depleted of the meiotic inhibitory kinase WEE-1.3.**A.K. Allen¹, R. Boateng¹, J. Nesmith², A. Golden²;¹Department of Biology, Howard University, Washington, DC, ²NIDDK/NIH, Bethesda, MD

Meiosis is the specialized cell cycle by which the haploid gametes, oocytes and sperm, are produced. Errors in meiosis can result in serious issues such as infertility, birth defects or tumorigenesis. Meiosis is controlled via dueling regulatory phosphorylation events on the cyclin-dependent kinase (Cdk) component of maturation promoting factor (MPF). The highly conserved Wee1/Myt1 family of kinases places inhibitory phosphorylations on Cdk that are necessary to keep MPF inactive and thus regulate the

progression of meiosis. Despite many years of study, in multiple animal systems, our understanding of meiosis is still incomplete. The studies described here in *Caenorhabditis elegans* are aimed to increase our knowledge regarding players in the meiotic pathway.

In *C. elegans* it was previously shown that depletion of the Myt1 ortholog, WEE-1.3, results in precocious oocyte maturation and infertility (Burrows *et al.*, 2006). We further characterized the precocious oocyte maturation phenotype observed upon WEE-1.3 depletion to better grasp why the oocytes are fertilization-incompetent. We will demonstrate here that WEE-1.3-depleted proximal germlines have begun to transcribe embryonic genes and the precocious oocytes inappropriately express proteins in patterns normally found in embryos. Secondly, we performed an RNA interference (RNAi) suppressor screen of the infertility phenotype exhibited upon WEE-1.3 depletion to identify new players involved in the meiotic pathway. We found 44 genes, that when co-depleted along with WEE-1.3, restore fertility to the animals. Many of these genes previously had no known role in reproduction or meiosis. Finally, we will report on the initial studies being conducted on one specific suppressor, ETR-1. ETR-1 is an RNA-binding protein that was previously characterized as being muscle-specific in the worm (Milne and Hodgkin, 1999). However, homologs in other organisms, including humans, have reproductive roles and mutations in some of the homologs have been linked to fertilization defects. Our data indicates a novel role for ETR-1 in the somatic gonad that ultimately influences oocyte maturation. We are actively investigating the mechanism by which depletion of ETR-1 suppresses WEE-1.3 depletion.

As our studies of ETR-1 are alluding to, many of the genes identified in the RNAi suppression screen have the potential to be unidentified players in both the meiotic and mitotic cell cycles. Thus these studies are providing valuable input into how the cell cycle is appropriately regulated.

P2320

Board Number: B1001

A role for dynein light chain in germline stem cell maintenance in *C. elegans*.

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PUF family RNA binding proteins are conserved stem cell regulators in *Drosophila* and planaria (Lin and Spradling, 1997; Salvetti *et al.*, 2005). FBF-1 and FBF-2, two similar PUF family translational repressors, are required for maintenance of germline stem cell in *C. elegans* (Crittenden *et al.*, 2002). Here we report identification of dynein light chain DLC-1 as a specific cofactor of FBF-2, but not FBF-1. In germline stem cells, FBF-2 localizes to perinuclear germ granules or P granules in *C. elegans* (Voronina *et al.*, 2012). This localization is important for FBF-2 binding to target mRNAs and its activity as a translational repressor. By contrast, FBF-1 does not localize to P granules, and its function does not depend on P granule integrity. The goal of this study was to investigate mechanisms of FBF-2 localization to P granules. DLC-1 is an LC8-type light chain, a cargo-binding component of dynein motor complex. Dynein traffics organelles, proteins, and mRNAs toward the minus ends of microtubules. Knock-down of *dlc-1* by

RNAi correlates with loss of FBF-2 perinuclear accumulation even when perinuclear P granules are not affected and also causes a decrease in FBF-2 activity as a translational repressor. By contrast, FBF-1 localization is not affected by *dlc-1* knock-down, and FBF-1 remains able to regulate its targets. In vivo, FBF-2 is found in a complex with DLC-1 by co-immunoprecipitation. In the in vitro pulldown assay, DLC-1 interacts with FBF-2, but not FBF-1, suggesting that specificity of DLC-1 contribution to FBF-2 function is based on selectivity of protein-protein interactions. Interaction between DLC-1 and FBF-2 depends on the regions of sequence divergence between FBF-2 and FBF-1.

We hypothesize that DLC-1 binds FBF-2 and promotes its localization to P granules, which is important for FBF-2-mediated translational regulation and germline stem cell maintenance. Dynein motor complex has been implicated in formation, transport, and dynamics of RNA granules in several cell types. Our findings suggest that LC8-type light chains such as DLC-1 may directly regulate recruitment of specific components to the RNA granules in stem cells.

P2321

Board Number: B1002

A novel function for CDK--11 in fertilization and fecundity in *C. elegans*.

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Functional germ lines produce gametes that are capable of producing viable offspring. When gamete development is disrupted, a reduction in fecundity may occur, with sterility being a potential outcome. The *C. elegans* hermaphrodite produces both oocytes and sperm and is capable of self-fertilization. In the worm, fertility is dependent on the interdependent functioning of the germ line tissue and the surrounding somatic cells (sheath cells). CDK-11 is a conserved kinase with established roles in transcription, centrosome duplication, microtubule nucleation, and apoptosis. *C. elegans* possesses two *cdk-11* genes, (*cdk-11.1* and *cdk-11.2*) and mutation in either produces a significant reduction in brood size. The mutation of *cdk-11.1*, causes gross morphological defects of both the oocytes and sperm in hermaphrodites, whereas *cdk-11.2* mutant hermaphrodites have normal gamete morphology. Transcriptional and translational reporter constructs demonstrate that both *cdk-11* homologs are expressed throughout the entire germ line and also in the sheath cells. However, localization of endogenous CDK-11.2 is found primarily in the meiotic cells of the germ line and in the nuclei of the sheath cells. Although mutant CDK-11.2 is expressed, CDK-11.2 fails to localize normally in the germ line. In addition to gamete defects, CDK-11 mutants also show a reduction in laying rates when compared to wild type worms. In summary, our data suggests that CDK-11 is required for the later stages of gamete production and possibly also plays a role in ovulation.

P2322**Board Number: B1003****A protein complex directs assembly of the vitelline layer of the *C. elegans* eggshell.**D. Partida¹, Z. Wilson¹, H. Lamb¹, S. Olson¹;¹Biology, Pomona College, Claremont, CA

Fertilization triggers rapid remodeling of the zygote surface to create an extracellular barrier that protects the developing embryo and prevents polyspermic fertilization. In nematodes, this barrier is a multi-layered eggshell that assembles in a hierarchical manner. The outermost layer of the eggshell is called the vitelline layer, which is present on the oocyte before fertilization. Immediately following fertilization, the vitelline layer lifts off the oocyte surface and provides a first line of defense. Despite its importance, little is known about the composition of the vitelline layer or how it is remodeled to form the outermost eggshell layer. A previous RNAi screen in the nematode *C. elegans* identified PERM-2 and PERM-4 as proteins required for eggshell formation. PERM-2 and PERM-4 were found to localize to the vitelline layer and were co-dependent for proper localization. PERM-2 and PERM-4 localization also depended on CBD-1, a protein required to stabilize a complex involved in the oocyte-to-embryo transition. The eggshell still forms when PERM-2/4 are depleted, but the shell is more porous as measured by loss of a soluble protein that normally resides in the perivitelline space.

P2323**Board Number: B1004****The small GTPase Arf6 is essential for sea urchin embryogenesis.**P. Ahiakonu¹, J.L. Song¹, K. Landis,¹ M. Dumas¹;¹Biological Sciences, University of Delaware, Newark, DE

Department of Biological Sciences, Newark, DE 19716 Arf6 is a small GTPase that acts as a molecular switch, cycling between the plasma membrane in its active GTP bound form and endosomal compartments in its inactive GDP bound form. While a rich knowledge exists in the cellular functions of Arf6, relatively little is known about its physiological role in development. This study examines the function of Arf6 in early development using the purple sea urchin as a model. We found that perturbation of Arf6 in the form of its knockdown, the constitutively activated GTPase defective Arf6 mutant Arf6 (Q67L), and the dominant active Arf6 mutant Arf6 (T27N), resulted in severe gastrulation defects. We tested perturbation of Arf6 during gastrulation by examining its effect in regulating three main types of cells that undergo critical cell movement during gastrulation: endodermal cells that undergo morphogenesis to form the larval gut, primary mesenchyme cells (PMCs) that undergo migration to give rise to the larval skeleton, and pigment cells that are highly motile to engulf potential pathogens. Arf6 knockdown led to a range of dose-dependent severity of developmental defects including a delay in development, vegetal cell attachment, and exogastrulation. Results indicate that Arf6 perturbed embryos have defective larval gut, aberrant PMC patterning and skeletal structures, and

pigment cell morphological changes. Overall our results demonstrate that Arf6 is essential for proper morphogenic and cell movements of endodermally and mesodermally-derived cell types during early development.

P2324

Board Number: B1005

The role of the TORC1 regulator IML1 in meiotic progression and the response to cellular stress.

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Under conditions of nutrient stress, yeast cells enter the meiotic cycle and begin sporulation. Target of Rapamycin Complex 1 (TORC1) is a central regulator of growth and metabolism, and its activity needs to be down regulated for yeast to sporulate, linking TORC1 activity to progression into meiosis. In metazoans, it is poorly understood how metabolic inputs influence meiotic progression and gametogenesis. The IML1/GATOR1 protein complex has been shown to down regulate TORC1 activity in times of nutrient stress, and we find that knocking down IML1 expression in the *Drosophila melanogaster* germline delays the oocyte's entry into meiosis suggesting TORC1 needs to be inhibited for meiotic entry during *Drosophila* oogenesis. Conversely, the SEA/GATOR2 complex components Mio and Seh1 are required to oppose the TORC1 inhibitory activity of the GATOR1 components Nprl2 and Nprl3 in the female germline but are not required for the development and/or growth of many somatic cell types. Our data support the model that the central importance of the SEA/GATOR complex in the regulation of TORC1 activity during gametogenesis has been conserved from single celled organisms to multicellular eukaryotes.

P2325

Board Number: B1006

Identification of a receptor pair in the gamete membrane adhesion step during cell-cell fusion in *Chlamydomonas*.

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Gamete fusion during fertilization is essential to produce a new organism, yet the molecular mechanisms of the gamete membrane fusion reaction are poorly understood. HAP2, a single pass transmembrane protein, is the only broadly conserved protein known to be essential during fertilization in protists, alga, higher plants and several multicellular animals. Previously, we established that 1) the membrane protein FUS1 on plus gametes is essential for membrane adhesion during the membrane fusion reaction between plus and minus *Chlamydomonas* gametes and 2) that HAP2 in minus gametes is essential for membrane merger per se after species-specific membrane adhesion. Here, we report

identification of a membrane adhesion receptor on minus gametes (MAR1) that binds to FUS1. MAR1-FLAG forms a complex with FUS1-HA in mixtures of lysates of MAR1-FLAG minus gametes and FUS1-HA plus gametes. RNA-Seq shows that MAR1 transcripts are specifically expressed in minus gametes and substantially upregulated during gamete activation, consistent with anti-MAR1-FLAG immunoblotting results. MAR1 specifically localizes to the apical membrane patch on minus gametes specialized for membrane fusion. Preliminary knockdown experiments indicate that MAR1 is essential for membrane adhesion. To our surprise, MAR1 also binds to HAP2 in minus gametes, indicating that proteins required for membrane adhesion interact with the protein required for membrane fusion. The discovery of the MAR1-FUS1 receptor pair along with knowledge that HAP2 is essential for membrane fusion now takes Chlamydomonas to the forefront of our understanding of the molecules required for eukaryotic fertilization. This work was supported by NIH GM56778.

P2326

Board Number: B1007

The Drosophila SEA/GATOR complex controls meiotic progression and the response to amino acid starvation by modulating TORC1 activity.

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In single-celled eukaryotes, the pathways that monitor nutrient availability are central to regulating the meiotic program and spore development. However, how metabolic inputs influence meiotic progression and gametogenesis remains poorly understood in metazoans. Here we define opposing functions for the highly conserved SEA/GATOR complex members Nprl2/Nprl3 and Mio/Seh1 in the regulation of TORC1, a master regulator of metabolism, during Drosophila oogenesis. We demonstrate that Nprl2/Nprl3 inhibit TORC1 activity in response to amino acid starvation and are critical to oocyte survival during times of protein scarcity. Conversely, Mio/Seh1 positively regulate TORC1 and oppose the activity of Nprl2/Nprl3. Independent of nutrient status, in mio and seh1 mutants the constitutive inhibition of TORC1 in the female germline results in the activation of autophagy and a block to oocyte growth and development. In contrast, mio and seh1 have minimal effects on the growth and development of somatic tissues. Thus, in Drosophila the requirement for the TORC1 activating components of the SEA/GATOR complex is tissue specific. Our data support the model that the central importance of the SEA/GATOR complex in the response to amino acid starvation and the regulation of TORC1 activity during gametogenesis have been conserved from single celled to multicellular eukaryotes.

P2327**Board Number: B1008****Orb and Wispy cooperate in the cytoplasmic polyadenylation of gurken (*grk*) mRNA during *Drosophila* oogenesis.**K. Randolph¹, L. Thompson¹, A. Norvell¹;¹Biology, Coll New Jersey, Ewing, NJ

During *Drosophila* oogenesis, the TGF- α like protein Gurken (Grk) is required for establishing both of the major axes of the egg and future embryo. Consequently, Grk protein distribution within the ovary is tightly regulated, both temporally and spatially. It is well established that Grk protein distribution in the oocyte is regulated by a combination of the localization of the *grk* transcript and regulation of *grk* mRNA translation. While it is clear that *grk* translation is both repressed, when the mRNA is not properly localized and activated, once the transcript has reached its destination in the dorsal anterior corner of the oocyte, the molecular mechanism(s) of *grk* mRNA translational control have not yet been fully elucidated. Modulating polyA tail length is one mechanism for regulating translation of other mRNAs and is accomplished through cytoplasmic deadenylation and polyadenylation of transcripts. We have therefore investigated whether translational control of *grk* RNA is achieved through alterations in polyA tail length. Genetic evidence has shown that the cytoplasmic polyadenylation element binding (CPEB) protein Orb is required for appropriate Grk protein accumulation, suggesting that cytoplasmic polyadenylation of *grk* mRNA may have a role in its translational activation. Using a PCR-based polyadenylation assay, we have analyzed *grk* transcript polyadenylation status directly. We have found that *grk* polyA tail length is indeed shorter in transcripts isolated from *orb* mutant females. Additionally, females carrying mutations in *wispy* (*wisp*), a GLD-2 polyA polymerase lay eggs with slight patterning defects that are consistent with suboptimal levels of Grk protein at the D/A corner of the oocyte. *grk* mRNA is also underadenylated in ovaries from *wisp* mutant mothers. Finally, mutations in *wisp* enhance the ventralizing effects of a dominant negative *orb* allele, suggesting that Orb and Wisp function together in the cytoplasmic polyadenylation of *grk* mRNA once it has reached the dorsal anterior corner of the oocyte. Our current efforts are aimed at assessing whether the translational repression of unlocalized *grk* mRNA is also achieved through regulated deadenylation-adenylation. Initial experiments indicate that translationally repressed *grk* transcripts, for example in *spnF* oocytes or associated with the RNA-binding protein Squid (Sqd), are underadenylated. These findings suggest that regulation of Grk protein accumulation is, in part, achieved through polyA tail length alterations.

P2329**Board Number: B1010****Significance of testis specific PP1 γ 2 and its stoichiometric requirement for mammalian male reproduction.**T. Dudiki¹, S. Sen², N. Sinha², D.W. Kline², S. Vijayaraghavan²;¹Biomedical Sciences, Kent State University, Kent, OH, ²Biological Sciences, Kent State University, Kent, OH

Serine/threonine phosphatase family constitutes PP1, PP2A, PP2B and PP2C. PP1 has four isoforms PP1 α , PP1 β , PP1 γ 1 and PP1 γ 2 encoded by three genes. The isoforms PP1 γ 1 and PP1 γ 2 are splice variants of *Ppp1cc* gene, which is comprised of 8 exons. PP1 γ 2 arises due to splicing of intron 7 that is retained in PP1 γ 1. These two isoforms are identical except for the 22 amino acid C-terminus tail of PP1 γ 2 encoded by exon 8. While PP1 γ 1 is ubiquitous in somatic cells including Sertoli cells of testis, it is absent in developing male germ cells and spermatozoa. PP1 γ 2 is expressed at high levels exclusively in developing male germ cells of mammals. Non-mammalian spermatozoa may contain PP1 α or PP1 γ 1. The only phenotype observed in *Ppp1cc* knockout mice is male sterility due to impaired spermiogenesis. Mice with one allele of *Ppp1cc* gene (*Ppp1cc* +/-) however showed only a 28% decrease in PP1 γ 2 protein levels with no effect on sperm function or fertility indicating a dosage compensation or feedback mechanism regulating the levels of PP1 γ 2 in testis. Recent transgenic rescue studies in our laboratory support this hypothesis by showing a threshold level requirement of PP1 γ 2 to restore spermatogenesis and fertility in *Ppp1cc* null mice (PLoS One. 2012; 7(10): e47623). To study the effects of PP1 γ 2 over expression, we generated mice homozygous for the testis specific PP1 γ 2 transgene over wild type (*Ppp1cc* +/+) but observed no phenotype. Molecular analysis revealed additive levels of PP1 γ 2 mRNA without increase in protein levels. This indicates that PP1 γ 2 enzyme levels are tightly regulated and it's over expression could be unfavorable for normal functioning of testis or spermatozoa. However we could successfully express PP1 γ 1 in addition to PP1 γ 2 in developing germ cells by incorporation of testis specific PP1 γ 1 transgene (PP1 γ 1 cDNA lacking intron 7) in *Ppp1cc* +/+ or *Ppp1cc* +/- mice. PP1 γ 1 was also observed in sperm along with PP1 γ 2 but by displacing a portion of PP1 γ 2. The resulting phenotype was reduced male fertility and altered sperm motility characterized by decreased flagellar beat and reduced velocity. The results so far indicate two possible mechanisms regulating the PP1 γ protein levels in male germ cells: 1) expression of PP1 γ 2 by elimination of PP1 γ 1 via splicing and miRNA mediated degradation and 2) a translational regulation mechanism to maintain threshold levels of PP1 γ 2. Evolution of PP1 γ 2 and the mechanisms regulating the PP1 expression in testis appear to be essential for reproductive success of mammals. (Supported by R15HD068971)

P2330**Board Number: B1011****Effects of metabolic status caused by maternal obesity and/or high-fat diet intake on steroidogenic capacity of Leydig cells in adult rats.**M.E. Pinto¹, V. Reame², E.Z. Pytlowanciv³, A. Protzek², A. Rafacho⁴, D.L. Ribeiro⁵, S.R. Taboga⁶, R.M. Goes⁷;¹IBILCE/UNESP, S J Rio Preto, Brazil, ²IBILCE/UNESP, São José Do Rio Preto, Brazil, ³Univ Estadual De Campinas (Unicamp), São José Do Rio Preto - SP, Brazil, ⁴Physiological Sciences, Federal University of Santa Catarina, UFS, Florianópolis, Brazil, ⁵Histology, Institute of Biomedical Sciences/ Federal University of Uberlândia- UFU, Uberlândia, Brazil, ⁶Biology, São Paulo State University, IBILCE/UNESP, S J do Rio Preto, Brazil, ⁷Biology, São Paulo State University, IBILCE/UNESP, São José Do Rio Preto, Brazil

This investigation evaluates the effects of metabolic status caused by maternal obesity (MO) and/or high-fat diet (HFD) intake on steroidogenic capacity of Leydig cells (LC) in adult rats. MO was induced in female rats before pregnancy by HFD (20% lipids) for 15 weeks whereas control females received balanced diet (4% lipids). Male offspring resulting from these matings were divided in groups: Control (balanced diet from gestation to adulthood), O1 (MO during gestation), O2 (MO during gestation/lactation), O3 (HFD from weaning to adulthood), O4 (MO during lactation + HFD from weaning to adulthood) and O5 (MO during gestation/lactation + HFD from weaning to adulthood). Animals were euthanized at 18-week-old and biometric, metabolic and hormonal parameters were determined. Testes were evaluated for ultrastructural analysis, immunohistochemistry and western blotting for 17 β -hydroxysteroid dehydrogenase (17 β -HSD) and aromatase. Body weight, adiposity index and serum leptin levels indicate that all groups were obese, except for O1. Three progressive levels of impaired metabolic status were observed: O1 presented insulin resistance, O2 were insulin resistant and obese, and groups O3, O4 and O5 were insulin resistant, obese and diabetic. These three levels of metabolic damage were proportional to the increase of leptin and insulin, and decreased serum and intratesticular levels of testosterone. Moreover, there was a trend of increase of serum estrogen levels in O2, O3 and O4, and a drastic augment in O5, whereas the intratesticular levels decreased in all these groups. A low expression of 17 β -HSD was detected to all obese groups and a slight reduction of aromatase content was observed in all groups, mainly in O5. Testicular ultrastructural analyses revealed that in O3, O4 and O5 presented accumulation of myelin vesicles in the mitochondrial matrix. These figures were generally related to structural disorganization, increased mitochondrial size and signs of degeneration. In conclusion, MO and/or HFD intake reduces the steroidogenic capacity of LC, either by failures in the differentiation of these cells or due to effects of metabolic disorders arising from obesity, regardless the period of testis development. In addition, MO during gestation and lactation is as detrimental to steroidogenic capacity of LC as the exposure to HFD ingested in other stages of development. Financial Support: FAPESP.

P2331**Board Number: B1012****Maternal dazap2 regulates germ granule formation via inhibition of Dynein in zebrafish primordial germ cells.**M.M. Forbes¹, S. Rothhaemel¹, A. Jenny¹, F.L. Marlow^{2,3};¹Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, NY, ²Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, NY, ³Dominick P. Purpura Dept of Neuroscience, Albert Einstein College of Medicine, Bronx, NY

Primordial germ cells (PGCs) are the progenitor cells that give rise to the gametes. In some animals the germline is induced by zygotic factors whereas in others, like zebrafish, PGC specification occurs via the inheritance of maternal determinants known as germ plasm. In these animals, the germ plasm is packaged into ribonucleoprotein (RNP) complexes that are localized and protected in the oocyte and later promote the germ cell fate in the embryo. Within the PGCs germ plasm components localize to perinuclear granular-like structures that are analogous to the germ granules of other species. Although zebrafish PGC germ granules have been studied, the maternal factors regulating their assembly and contribution to germ cell development are not known. Here we identified the scaffold protein Dazap2 as a binding partner of Bucky ball, an essential regulator of oocyte polarity and germ plasm assembly. We show that eGFP-Dazap2 protein colocalizes with the germ plasm in primary oocytes and in the PGCs of embryos. We generated dazap2 maternal-effect mutants (Mdazap2) and identified a specific requirement for MDazap2 in germ granule formation in PGCs. Overexpression of eGFP-dazap2 enlarges germ granules of wild-type and rescues germ granule formation in Mdazap2 PGCs. We show that inhibiting Dynein in Mdazap2 mutants restores germ granule formation, revealing a novel role for dazap2 in regulating germ granule formation by a mechanism that involves Dynein inhibition. Taken together our loss of function and stable transgenic studies indicate that maternal Dazap2 colocalizes with the germ plasm in oocytes, likely via interaction with Buc, where it is sequestered until it is necessary to promote germ granule formation in the embryo.

P2332**Board Number: B1013****Effect of a combination of PTEN and PI3K inhibitors on superovulation in A/J mice.**O. Suzuki¹;¹National Institute of Biomedical Innovation, Ibaraki-shi, Japan

[Aim] Strain and individual differences in superovulation constitute a serious problem in mice. My preliminary study reported in ASCB2013 and AALAS2014 meetings suggested that an inhibitor (dipotassium bisperoxo (picolinato) oxovanadate (V), bpV(pic)) of Phosphatase and Tensin Homolog Deleted from Chromosome 10 (PTEN) on the same day for equine chorionic gonadotropin (eCG) and an

inhibitor (LY294002) of Phosphoinositide 3-kinase (PI3K) on the next day for eCG tended to increase the number of ovulated oocytes at superovulation by eCG and human chorionic gonadotropin (hCG). This study tested whether a combination of both PTEN and PI3K inhibitors promotes superovulation by gonadotropins. [Method] Five i.u. of eCG was intraperitoneally injected to 28-day-old A/J female mice. In “control (C)” group, only vehicle, no inhibitors, was given. In “bpV(pic)-only (B)” groups, 2 mg/kg of bpV(pic) dissolved in Ringer’s solution were given on the same day for eCG. In “LY294002-only (L)” group, 0.1 mg/kg of LY294002 dissolved in Ringer’s solution with 5% DMSO were given on the next day of eCG. In “both (BL)” group, both inhibitors were given on their respective days. Ovulation was induced by 5 i.u. of hCG 48 h after eCG injection. The numbers of oocytes ovulated were counted 16 h after hCG injection. After normality and equal variance were confirmed by Shapiro-Wilk and Levene tests, respectively, pairwise multiple comparisons of average numbers of ovulated oocytes between groups were performed by Tukey tests. [Results] The average numbers of oocytes collected were 7.8 ± 3.0^a , 22.8 ± 3.2^a , 10.2 ± 3.7^{bc} , and 15.0 ± 1.7^{ac} (mean \pm SEM) in groups C, B, L and BL, respectively (n=5 in C, B, and L; n=6 in BL). Values with different superscripts are significantly different ($p < 0.05$). Significantly more oocytes were collected in bpV(pic)-only group (B) than in control (C). More oocytes tended to be collected in LY294002-only group (L) than in control (C), but LY294002 suppressed the beneficial effect of bpV(pic) on superovulation when both bpV(pic) and LY294002 were administered (group BY). [Discussion] This new method using both PTEN inhibitors and gonadotropins is effective for improving superovulation efficiency, although the optimal method to modulate PI3K signaling pathway in the mouse ovary (e.g., use of PI3K inhibitors) should be determined. This work was supported by a grant from the Ministry of Health, Labour and Welfare of Japan.

P2333

Board Number: B1014

Maternal protein restriction: Study of the seminiferous epithelium of rat pups weaned.

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Maternal protein restriction may cause changes morphophysiology of the male reproductive organs of offspring and other organs during later life. The androgens are essential to ensure normal development of the male reproductive organs in a phase named of fetal masculinization programming window. This study analyzed the effects of maternal protein restriction during pregnancy and lactation period on development of the rat pups testis. Pregnant rats were divided in two experimental groups (n=10/group): Hypoproteic group fed with hypoproteic diet (6% protein) and control group normal diet (17% protein) and the offspring rats were evaluated at 21 days old. The testes were studied through immunohistochemistry analysis to evaluate the expression of Ki-67. Sections of the testis were stained

immunohistochemically using the avidin-biotin-peroxidase complex method. The body and testis weights were determined. The spermatogonia population positive Ki-67 was counted in 20 seminiferous tubules per rat. None alteration in the numbers of positive Ki-67 spermatogonia was observed. On the other hand, there were reductions of body and testis weights in the hypoproteic group. The seminiferous tubules of control group showed spermatogonia, Sertoli cells and primary spermatocytes organized in concentric layers. In the hypoproteic group, the epithelial cells population showed diffuse organization. In the both groups, part of primary spermatocytes been identified in the lumen of seminiferous tubules, indicating cellular migration. We concluded that maternal protein restriction causes alterations in the organization of seminiferous epithelium of pups weaned. Research supported by FAPESP (Grant #2011/09110-8; 2013/14886-0) and FUNDUNESP (Grant #696/11).

P2334

Board Number: B1015

Gap junctions of porcine COCs (Cumulus-Oocyte Complexes) and nuclear maturation are inhibited by Perfluorooctane Sulfonate (PFOS) during in vitro maturation.

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Perfluorooctane sulfonate (PFOS) is a persistent and bio-accumulative pollutant ubiquitous in wildlife and humans. Studies have shown that PFOS inhibit Gap Junction Communication (GJC) in somatic cells, and as levels of PFCs are found in human follicular fluid, the aim of the present study was to analyze the effects of PFOS on COCs GJC and the in vitro viability and maturation of pig oocytes. Cumulus-oocyte complexes were cultured in maturation medium supplemented with 0 (control), 12.5, 25 and 50 μM PFOS.

Results show that oocyte viability decreased in PFOS-exposed oocytes in a concentration-dependent manner with a lethal concentration (LC50) of 29.8 μM . Oocyte maturation was also inhibited in a concentration dependent manner with a maturation index (MI50) of 9.9 μM .

Gap junction communication was blocked in COCs exposed to 9.9 μM after 4.5 h. Nevertheless, connexin 45 (Cx45), Cx43 and Cx60 expression was not affected.

These findings show a relevant effect of PFOS on the in vitro viability and maturation of pig oocytes, which might be based on the early blockage of GJC in COCs.

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Cell Fate Determination

P2335

Board Number: B1016

Transmitotic persistence of Wnt pathway activity diversifies gene expression in *C. elegans* embryos.

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Quantitative differences in signaling pathway activity are a powerful potential mechanism for diversifying cell fates during development. However, few examples of this mechanism have been identified *in vivo*, in part due to the challenges of performing quantitative assays. The objective of this study was to evaluate whether *C. elegans* embryos use quantitative differences Wnt pathway activity to regulate gene expression. The Wnt signaling pathway plays a conserved role during animal development, transcriptionally regulating distinct targets in different stages and cell types (i.e. contexts). This dependence of targets on context could reflect not only interactions with differentially expressed transcription factors, but also context-specific differences in the activity of the Wnt pathway itself. We investigated the role of Wnt pathway activity in target expression by using time-lapse microscopy and automated lineage tracing of *Caenorhabditis elegans* embryos to quantify expression of Wnt ligands, target genes, and nuclear localization of transcriptional effectors *in vivo* at single cell resolution throughout development. We measured the Wnt pathway-dependence of candidate targets and identified over a dozen important developmental regulators as new Wnt targets. We found that most targets require the Wnt-effector transcription factor POP-1/TCF for either activation or repression but not both. Contrary to existing models, we observed that Wnt-mediated transcriptional activation is strongest in cells that received a Wnt signal in two or more consecutive divisions. We found that these repeatedly signaled cells have higher nuclear β -catenin concentrations and are more likely to express targets that require POP-1 for transcriptional activation. Taken together, these results suggest that the persistence of Wnt signaling across mitosis can integrate lineage history and allow Wnt to activate distinct targets in different developmental contexts.

P2336**Board Number: B1017****Actomyosin contractility is required for Notch-mediated patterning of sensory organ precursor cells in the *Drosophila notum*.**G.L. Hunter¹, B. Baum², G. Charras³;¹MRC Laboratory for Molecular Cell Biology, University College London, London, United Kingdom,²Laboratory for Molecular Cell Biology, University College London, London, United Kingdom, ³London Centre for Nanotechnology, University College London, London, United Kingdom

The differentiation of sensory organ precursor (SOP) cells in the *Drosophila notum* epithelium is an excellent model system in which to study long-range lateral inhibition via Notch signaling. Previous research showed that robust, tissue-level organization of SOPs requires a population of basal, actin-based protrusions. **Here, we investigate whether forces generated by the cytoskeleton also play a role in the process of long-range lateral inhibition.** (1) We identify a dynamic pool of non-muscle myosin II heavy chain, together with phosphorylated myosin regulatory light chain, which localizes to the basal cytoskeleton of the notum epithelium. (2) Modulation of myosin II activity via the overexpression of phospho-mimetic or phospho-dead myosin regulatory light chain alters basal protrusion dynamics (e.g., retraction and extension rates) and morphology (e.g., branching and lamellopodia) without affecting their length. (3) Decreased myosin II activity, induced through the expression of dominant negative constructs, (a motor-less heavy chain or a phospho-dead regulatory light chain), leads to defects in long-range lateral inhibition, indicated by the decreased spacing of SOP and SOP-like cells in the notum. (4) Moreover, these changes in signaling are not accompanied by striking changes in the localization of Notch or Delta protein. Together these results suggest that actomyosin-based forces play a role in activation of Notch signaling. (5) To investigate how myosin II activity affects the Notch signaling pathway to induce these changes in tissue patterning, we performed live, quantitative analysis of Notch signaling in the notum, using a novel Notch signaling reporter (He and Perrimon, unpublished). Using this tool, we can demonstrate that Notch signaling dynamics in the notum are consistent with previous mathematical models of lateral inhibition, in which cells undergo dynamic changes in their state as they commit to an epithelial or SOP fate. (6) We find that the expression of phospho-dead regulatory light chain results in a shift in the kinetics of the Notch signaling response in notum epithelial cells, and suggests that Notch activation is delayed in the absence of myosin II contractility relative to control. These data suggest that actomyosin contractility plays a key role in the activation of Notch signaling during notum patterning. Thus, protrusions may mediate long range, mechanically-induced Notch signaling in vivo – as previously proposed for Notch-Delta signaling in vitro. This identifies a mechanism by which cells can integrate mechanical forces and cell signaling to drive developmental processes.

P2337**Board Number: B1018****Genome-wide demethylation by 5-aza-2'-deoxycytidine alters the cell fate of stem/progenitor cells.**Z. Hu¹, Y. Zhou²;¹Department of Otolaryngology-HNS, Wayne State Univ, Detroit, MI, ²Wayne State Univ, Detroit, MI

DNA methyltransferase (DNMT) inhibitor 5-aza-2'-deoxycytidine (5-aza-CdR) is able to cause DNA demethylation in the genome and induce the expression of silenced genes. Whether DNA demethylation can affect the gene expression of stem/progenitor cells has not been understood. Mouse utricle epithelia-derived progenitor cells (MUCs), which possess stem cell features as previously described, exhibit a potential DNA methylation status in the genome. In this study, MUCs were treated with 5-aza-CdR to determine whether DNMT inhibitor is able to induce the differentiation of MUCs. With 5-aza-CdR treatment for 72 hr, MUCs expressed epithelial genes including *Cdh1*, *Krt8*, *Krt18*, and *Dsp*. Further, hair cell genes *Myo7a* and *Myo6* increased their expressions in response to 5-aza-CdR treatment. The decrease in the global methylated DNA values after 5-aza-CdR treatment indicated a significant DNA demethylation in the genome of MUCs, which may contribute to remarkably increased expression of epithelial genes and hair cell genes. The progenitor MUCs then turned into an epithelial-like hair cell fate with the expression of both epithelial and hair cell genes. This study suggests that stem cell differentiation can be stimulated by DNA demethylation, which may open avenues for studying stem cell fate induction using epigenetic approaches.

P2338**Board Number: B1019****Shaping the BMP morphogen gradient through the spatiotemporal regulation of metalloprotease activity in the zebrafish embryo.**F. Tuazon¹, M.C. Mullins¹, J. Zinski¹, D. Umulis², Y. Huang²;¹Department of Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA,²Agricultural and Biological Engineering, Purdue University, West Lafayette, IN

Morphogens are key developmental signaling molecules that specify distinct cell fates in a concentration-dependent manner. The regulation of morphogen signaling across space and time is fundamental to proper tissue patterning, yet how these regulatory mechanisms function *in vivo* remains unknown. Bone Morphogenetic Proteins (BMPs) act as a morphogen to pattern dorsoventral (DV) axial tissues in invertebrates and vertebrates. Since BMPs also pattern the neural tube and developing limbs, understanding the spatiotemporal mechanisms that generate and shape BMP gradients is of great importance.

The shape of the BMP gradient spatially across the DV axis is critical: precise amounts of BMP signaling at discreet DV positions specify distinct cell fates. Since the DV axis is patterned from late blastula

through gastrula stages, the BMP gradient must also be maintained temporally through the cell movement process of gastrulation. Gastrulation generates the three embryonic germ layers, reorganizing a large number of cells in a short period of time and may challenge the mechanisms that shape the BMP gradient. To determine the shape of the BMP gradient, we developed a quantitative immunofluorescence assay of nuclear phosphorylated Smad1/5, a direct intracellular readout of BMP signaling. Using this assay, we discovered that the shape of the BMP gradient significantly changes during gastrulation: the BMP gradient steepens both dramatically and rapidly between mid- and late gastrulation stages.

The spatiotemporal regulation of the shape of the BMP gradient requires extracellular modulators of the BMP ligand. An essential extracellular modulator is Chordin, a BMP antagonist that binds BMPs to inhibit signaling. Chordin is central to generating the initial BMP signaling gradient and continually regulates the BMP gradient during DV patterning. Chordin itself is regulated by two key classes of proteins: (i) the highly homologous metalloproteases Tolloid and Bmp1a, which cleave and inactivate Chordin, and (ii) the metalloprotease inhibitor Sizzled. We discovered that *tolloid* and *sizzled* mutants display distinct alterations in the shape of the BMP signaling gradient at the end of gastrulation, suggesting region-specific roles for Tolloid and Sizzled. Our data indicate that Tolloid maintains the steepness of the late BMP signaling gradient, while Sizzled broadly restricts BMP signaling in lateral regions. These results support a novel requirement for metalloprotease regulation from mid- to late gastrulation stages to steepen the BMP signaling gradient and correctly pattern the posterior tissues of the zebrafish embryo.

P2339

Board Number: B1020

Dissecting cell fate potential and cytoarchitectural dynamics in developing neural stem cells in vitro reveals functional correlates to human corticogenesis.

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Early neuroepithelial progenitors derived from pluripotent stem cells (PSCs) serve as a primary in vitro neural stem cell (NSC) source for generating neural cell type diversity. However, the heterogeneity of neuroepithelial cell cultures and their rapid transition through distinct neural stem and progenitor cell types impedes development of in vitro strategies for understanding lineage transition and neural cell type specification. This is further challenged by the lack of in depth cellular and molecular characterization of progenitor cells, together introducing a major challenge for harnessing their full potential in vitro. To tackle these limitations, here we dissected the transition of neuroepithelial cells through distinct potencies by prospective isolation of primary progenitor cells derived from human ES cells during long-term neural differentiation based on their Notch activation. We demonstrate that Notch activation in neuroectodermal cells is required for the establishment of neuroepithelial cells with

broad developmental potential and strong proliferation capacity. Notch active neuroepithelial cells rapidly progress into early and mid neurogenic cerebral radial glial (RG) cells followed by gliogenic RG in a Notch dependent manner. Transition through these cell types correlates with forebrain specification, cortical lamination and glial transformation at both functional and molecular levels. Furthermore, we found a functional correlation between Notch activation in early RG cells and their ability to form neural rosette structures - the in vitro counterpart of cortical RG cells. Mechanistically, Notch activation in RG cells confers a strong apicobasal cell polarity, which in turn enables their organization into rosette structures demarcating ventricular zone- and subventricular zone-like equivalents. We further demonstrate that rosette organization and Notch activation enable interkinetic nuclear migration and cell division at rosette apical sites, strongly suggesting that these features interplay to ensure the maintenance of neurogenic RG cell pools in vitro. Transcriptional analysis and subsequent shRNA screen validation reveal essential key factors involved in relaying Notch activation through neuroepithelial cell induction, radial glial cell transition and glial transformation. Our observations assign Notch activation and rosette formation as essential components orchestrating NSC ontogeny in vitro, by establishing identity of neuroepithelial cells, maintaining their numbers, and dictating their transition through distinct potencies. Our cellular and molecular observations provide a first insight into human NSC ontogeny and propose a well-controlled platform to dissect the development of normal and pathogenic NSCs and their progeny.

P2340

Board Number: B1021

Epithelia patterning and body plan mapping in the *Drosophila* egg chamber: cell fate specific screen.

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The precise establishment of the fate cells take to make up the different tissues of the body is fundamental to the development of all animals. A clear example of this is the patterning of the female germline of *Drosophila melanogaster*, which ultimately establishes the embryonic body plan of the animal, similar to vertebrates. A group of defined cells in the follicular epithelium encasing the germline, known as posterior follicle cells, are responsible for defining the first axis of the body by signalling their position to the oocyte. Only once this position is indicated to the oocyte can it go on to set the second axis of the body plan. To understand the process, whereby posterior follicle cells induce asymmetry in the oocyte, we designed a cell specific expression-profiling screen comparing the profile of posterior follicle cells to that of the remaining body of follicle cells. From this we identified a number of membrane proteins, transcription factors, G-protein receptors, and many genes that had been previously characterized in the follicular epithelium, as well as a small number having an unknown function in the fly. Three overlapping screens were employed to sift through the candidates from the expression-profiling screen and identify genes that have an essential function in the follicular epithelium: mutant allele, overlapping deficiency, and RNAi Screens. The results from these screens

culminated in the identification of a total of 9 candidate genes that have an interesting phenotype. Two of these, *midline* and *H15*, had recently been shown to be differentially expressed in the follicular epithelium and essential for patterning. The other top two interesting candidate genes, *Galpaf* and that of a previously undescribed transmembrane protein, display tissue architecture defects and oocyte polarizing defects, respectively, when knocked down. To fully characterize the function of these genes, both are currently in our pipeline to create flies with null mutations using the CRISPR/Cas9 system.

P2341

Board Number: B1022

Biophysical Properties of the Cellular Microenvironment Influence Hepatocyte Differentiation.

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Tissue-engineered liver constructs possess the potential to not only serve as cell-based therapeutics for treating liver disease, but they may also serve as model systems for drug toxicity analysis or the study of disease progression. Unfortunately, the utility of these liver constructs is limited, as current protocols for the differentiation of hepatocytes result in immature hepatocytes that fail to replicate the physiologic function of fully-differentiated adult hepatocytes. Furthermore, current *in vitro* culture methods fail to maintain hepatocyte function over extended periods of time. One possible means for addressing these issues is through manipulation of the biophysical microenvironment, which has been shown to play a significant role in the differentiation and functionality of numerous cell types. While previous studies have varied extracellular matrix coatings and substrate elastic modulus in order to optimize hepatocyte differentiation, little is known about the mechanism by which biophysical properties influence hepatic cell fate decisions. An understanding of this mechanism is key before the biophysical microenvironment can be properly engineered to generate functional liver constructs. In this study, we characterized changes in the biophysical microenvironment during the course of hepatocyte differentiation and began to identify a mechanism by which these properties influence hepatic fate determination. We used a multi-stage protocol for the differentiation of embryonic stem cell-derived hepatocytes during which cells were directed from a definitive endoderm stage, to specified hepatic lineage, to hepatoblast formation, to a final hepatocyte-like maturation state. Flow cytometry analysis demonstrated differentiation stage-specific integrin expression profiles during hepatic differentiation. In turn, these lineage-specific integrin expression profiles drove specific cell adhesion behaviors (cell attachment, cell spreading, actin organization, focal adhesion formation) in response to changes in substrate stiffness and extracellular matrix composition. Furthermore, changes in the biophysical

microenvironment significantly influenced the process of hepatocyte differentiation. These findings demonstrate that biophysical properties, such as substrate elastic modulus and extracellular matrix composition, play a vital role in hepatocyte differentiation. Additional studies are now underway to further identify the mechanism by which biophysical properties influence hepatic differentiation and to harness this insight in order to generate physiologically relevant tissue-engineered liver constructs for cell-based therapeutics, drug toxicity assays, and models of liver disease.

P2342

Board Number: B1023

Distinct Actin Nucleation Mechanisms are Required for the Differentiation Programs of EMT and Embryonic Stem Cells.

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Marked advancements have been made toward identifying transcriptional events controlling the programs of epithelial to mesenchymal differentiation (EMT) and embryonic stem cell (ESC) differentiation. However, both programs require dramatic remodeling of actin filaments and we know little about how this remodeling is regulated, particularly the role of actin nucleators that generate distinct actin architectures. We found that formins are necessary for EMT but the Arp2/3 complex is necessary for ESC differentiation. The broad spectrum formin inhibitor SMIFH2 but not the Arp2/3 complex inhibitor CK666 blocked EMT of human lung A549 cells and mouse mammary NMuMG cells induced by TGF- β . SMIFH2 blocked the change in cell shape to a mesenchymal morphology, remodeling of actin filaments to actin stress fibers, loss of cell-cell contacts, decreased expression of E-cadherin, increased expression of fibronectin and nuclear translocation of the transcription co-factor MRTF. We also found the surprising result that SMIFH2 inhibited proximal signaling by TGF- β , as indicated by decreased phosphorylation of Smad2 (pSmad2), a TGF- β receptor substrate. We tested shRNA for 9 of the 15 human formin family members and found that silencing expression of only two, FHOD1 and formin-1, inhibited EMT, but through distinct mechanisms. FHOD1 shRNA blocked the assembly of actin stress fibers and increased phosphorylation of myosin light chain that were rescued by expression of wild type FHOD1; however, pSmad2 was not blocked. Formin-1 shRNA blocked loss of cell-cell contacts, internalization of E-cadherin and nuclear translocation of MRTF but not the assembly of actin stress fibers. We are testing whether formin-1 is necessary for increased pSmad2. In contrast to EMT, CK666 but not the inactive analog CK689 or SMIFH2 inhibited mouse ESC differentiation. Using a dual reporter line with self-renewing, undifferentiated cells expressing mCherry and differentiated cells expressing GFP, we used FACS analysis to show that at 72 h of differentiation there were no differences between controls, CK689 or SMIFH2 but with CK666 there were 50% more self-renewing cells and 35% fewer differentiated cells. Differentiated cells had substantially more and larger membrane protrusions and distinct asymmetric clustered actin filaments at protrusions. These changes were completely blocked by CK666 but not SMIFH2. To our knowledge, neither Arp2/3 complex regulation of stem cell

differentiation nor formin-dependent Smad signaling has been reported. We predict that requirements for formin-dependent EMT compared with Arp2/3-dependent ESC differentiation reflect distinct needs of the differentiation program, specifically the need for increased cell contractility with EMT but not ESC differentiation.

P2343

Board Number: B1024

Neogenin as a receptor for the early cell fate determination in preimplantation mouse embryos.

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The first cell lineage determination in embryos takes place when two cell populations are set apart, each differentiating into the trophectoderm (TE) and the inner cell mass (ICM), respectively.

Positional/polarity cues trigger this differentiation, but it is unclear which receptor transduces extracellular cues into cell fate determination. We provide evidence for neogenin as an authentic receptor in preimplantation mouse embryos. A polarized and transient distribution of neogenin was manifested in early blastomeres. Neogenin up-regulation accelerated embryonic development concomitant with normal ICM development and activation of the ICM-specific genes Oct3/4, Sox2, and Nanog while its depletion by small hair-pin RNAs (shRNAs) caused a developmental arrest accompanied by poorly endowed ICMs and activation of the TE-specific genes Cdx2 and Tead4. Treatment with netrin-1 impaired both embryonic development and ICM formation while RGMc led to opposite consequences, demonstrating that neogenin helps guide blastomeres to the first cell fate determination relaying extracellular cues.

KEY WORDS: Neogenin, preimplantation mouse embryo, cell fate determination, netrin-1, RGMc

P2344

Board Number: B1025

Boc promotes neuronal differentiation by interaction with Abl and JNK activation.

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Neuronal development and differentiation are regulated by multiple coordinated events, such as cell migration, the outgrowth and guidance of axons and dendrites, and the accompanied neuron-specific

gene expression. A multifunctional protein, Boc belongs to the Ig/FNIII subfamily of cell surface receptor. It functions as a Shh coreceptor with the closely related protein, Cdo and another protein Gas1. Triple knockout mice for the Shh coreceptors exhibit phenotypes resembling the Hh signaling deficiency. In addition, Boc promotes myogenic differentiation as a component of multiprotein complexes containing related proteins Cdo and Neogenin, and a cell adhesion molecule N-cadherin. Boc plays a crucial role in the Shh-dependent axon guidance and synapse formation. Here we report that Boc promotes neuronal differentiation through interaction with Abl and JNK whereby activates JNK. Consistently Boc deficient neural stem cells exhibit defective neuronal differentiation and decreased JNK activation. Our data suggest that Boc regulates multiple events of neuronal differentiation including axon outgrowth and guidance.

P2345

Board Number: B1026

Investigating the Yeast Zygote Transcriptome.

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Zygote formation in *S. cerevisiae* provides opportunities to study cell membrane fusion, karyogamy, and organelle rearrangements. We have established a method for efficient zygote formation in physiological conditions, followed by flow cytometry-based purification (Zapanta Rinonos *et al.*, 2012). RNA extracts were then analyzed on microarrays (Affymetrix) to obtain transcriptome data. We studied replicate samples of haploid cells, diploid cells, and purified zygotes, as well as mating factor-stimulated cells. We analyzed each binary comparison of cell types and established a set of gene expression profiles to identify genome-wide transcriptome changes. A pioneering microarray analysis of mating factor-stimulated genes (Roberts *et al.*, 2000) shares 11 genes among the top 50 increased/decreased genes in our data set. A previous study combining ChIP, microarray, and binding motif analysis (cross-species consensus DNA sequences in fungi) identified a group of cell type-specific genes for haploid and diploid cells (Galgoczy *et al.*, 2004). We also identified 80% of these genes as being specific in a/alpha or haploid/diploid comparisons. Three groups of zygote-specific genes were identified by mapping genes that are regulated during this process: (1) spindle pole body, (2) mitochondrial electron transfer chain, and (3) transcriptional regulators. We hypothesize that regulation of these groups of genes is pivotal for cell-cell fusion, zygote formation, and subsequent cell proliferation.

P2346**Board Number: B1027****Investigating mechanisms of spindle orientation by cell-cell signaling.**J.K. Heppert¹, B. Goldstein²;¹Biology, University of North Carolina, Chapel Hill, NC, ²Department of Biology, University of North Carolina, Chapel Hill, NC

Mitotic spindle orientation is a critical process during development and homeostasis, often establishing the positions of cells relative to key developmental or proliferative cues. In some cases, signaling between cells is required for accurate spindle orientation. However, how cell-cell signaling controls mitotic spindle positioning remains unresolved. We are investigating mechanistic links between signaling pathway components and the forces that position the mitotic spindle. Molecules in the Wnt signaling pathway and regulators of the motor protein dynein are necessary to orient the mitotic spindle of a dividing cell in the four-cell stage *C. elegans* embryo. We are establishing a system to examine the roles of these relevant players and determine how their localization may affect their functions during spindle orientation. We used Cas9/CRISPR triggered homologous recombination to insert genes encoding fluorescent proteins at the endogenous loci of genes in the Wnt signaling pathway. Live imaging of *C. elegans* four-cell stage embryos confirmed enrichment of DSH-2/Dishevelled at the cell contact between Wnt signaling and responding cells and further revealed that MOM-5/Frizzled is present at all cell contacts and becomes enriched at the contact between the Wnt signaling and responding cell over time. Disrupting Wnt signaling using *mom-2*/Wnt RNAi in these tagged strains resulted in a lack of local enrichment, suggesting that Wnt signaling is necessary for the localization of MOM-5/Frizzled and DSH-2/Dishevelled at this stage. We are also tagging candidates to test our hypothesis that Wnt signaling controls mitotic spindle orientation by the recruitment or activation of force generators at key cortical sites. We have endogenously tagged DNC-2/p50/dynamitin, a member of the dynactin complex. Using direct cell manipulations, we are testing the ability of a Wnt signaling cell to act as an instructional cue for spindle orientation through the local recruitment of microtubule regulators. Determining how proteins in the Wnt signaling pathway intersect with molecules that generate force to position the mitotic spindle will expand our mechanistic understanding of how cell-cell signaling can control spindle positioning.

P2347**Board Number: B1028****Tunable directed neurogenesis of neural stem cells via optogenetic activation of canonical Wnt signaling.**A. Rosenbloom¹, L. Bugaj², D. Schaffer¹;¹University of California, Berkeley, Berkeley, CA, ²Univ California-San Francisco, San Francisco, CA

Parkinson's and Alzheimer's disease are neurodegenerative diseases that affect 40 million people worldwide. Adult neural stem cells (NSC) have great potential as cell replacement therapies. The adult

NSC microenvironment is likely highly dynamic, with signaling molecules presented at modulating intensities and durations. The canonical Wnt signaling pathway, whose activation involves β -catenin stabilization, is involved in the neurogenic NSC fate; indeed, activation via Wnt3a can lead to robust neurogenesis. To mimic in vivo dynamic signaling, our lab has developed a tunable optogenetic system to modulate β -catenin signaling through the oligomerization of LRP6. We have observed that NSCs undergo neuronal differentiation in a light signal dosage-dependent manner, even in the presence of relatively low level fluctuations or cycling of dark and light pulses. We show the existence of a temporal “buffer” region within which the total number of photons, rather than signal fluctuation, determines the levels of neurogenesis. This type of measurement highlights the novel strengths of our optogenetic system in elucidating a more complete understanding of the molecular mechanisms by which canonical Wnt signaling regulates NSC fate, furthering efforts to harness NSCs for neuroregeneration.

P2348

Board Number: B1029

Nrf2 represses neuroectoderm differentiation during early lineage specification of human embryonic stem cells.

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Early nervous system development is regulated by sets of lineage-specific transcription factors (e.g. PAX6) that control the expression of many neuronal target genes. However, early events that precede lineage-specific transcription factor expression have not been well studied. Here, we show that Nrf2, a master regulator of antioxidant transcriptional responses, plays a key role in determining early cell fate. First, we profiled whole transcriptome from human embryonic stem cells (hESCs), neuroectoderm (NE) and mesendoderm (ME) cells using RNA-seq. Hierarchical clustering analysis based on the expression of Nrf2 target genes shows similarity between hESCs and ME cells. In contrast, NE cells exhibited low levels of Nrf2 target gene expression. Next, downregulation of Nrf2 activity preceded initiation of PAX6 expression during NE differentiation, suggesting that Nrf2 downregulation might be a prerequisite for NE fate. Consistent with this hypothesis, Nrf2 activation by chemical activators or gene overexpression was sufficient to suppress NE derivation, while further activating ME marker expression. In the opposite experiment, Nrf2 inhibition by shRNA promoted NE differentiation. Collectively, these data suggest that Nrf2 represses NE differentiation and Nrf2 downregulation is an early event that determines NE fate.

P2349**Board Number: B1030****Regulation of MKL1 via actin cytoskeleton dynamics drives adipocyte differentiation.**H. Nobusue^{1,2}, N. Onishi³, T. Shimizu⁴, E. Sugihara³, Y. Oki⁵, Y. Sumikawa⁵, T. Chiyoda³, K. Akashi⁶, K. Kano⁵, H. Saya³;¹Keio Univ Sch Med, Shinjuku-ku, Tokyo, Japan, ²College of Bioresource Sciences, Nihon Univ, Kanagawa, Japan, ³Keio Univ Sch Med, Tokyo, Japan, ⁴Department of Pathophysiology, Hoshi Univ, Tokyo, Japan, ⁵Nihon Univ, Fujisawa, Japan, ⁶Medicine and Biosystemic Science, Kyushu Univ, Fukuoka, Japan

Cellular differentiation is regulated through activation and repression of defined transcription factors. Adipocyte differentiation is regulated by gene expression of a master regulator such as peroxisome proliferator-activated receptor- γ (PPAR γ). A hallmark of differentiation is a pronounced change in cell shape, which is determined by dynamics of the actin cytoskeleton. Adipocyte differentiation is thus associated with a shift in the structures formed by filamentous (F) actin from stress fibers to cortical fibers. The relation between such reorganization of the actin cytoskeleton and the PPAR γ -mediated adipocytic differentiation program has remained unclear, however. Here, we show that regulation of the transcriptional coactivator MKL1 (megakaryoblastic leukemia 1) by actin cytoskeleton dynamics drives adipocyte differentiation mediated by PPAR γ . Exposure of preadipocytes to an adipogenic cocktail results in the rapid disruption of actin stress fibres as a consequence of downregulation of RhoA-ROCK signaling, which regulates the formation of actin stress fibers and focal adhesions, and the consequent increase in monomeric G-actin levels. The resulting increase in the amount of G-actin leads to the interaction of G-actin with MKL1, which prevents nuclear translocation of MKL1 and allows expression of PPAR γ followed by adipogenic differentiation. We further found that depletion of MKL1 can drive adipocyte differentiation in preadipocytes and even in nonadipogenic fibroblasts in the absence of an adipogenic cocktail, and these data revealed that MKL1 functions as a gatekeeper that controls adipocyte differentiation. Moreover, we found that MKL1 and PPAR γ act in a mutually antagonistic manner in the adipocytic differentiation program. Our findings thus provide new mechanistic insight into the relation between the dynamics of cell shape and transcriptional regulation during cellular differentiation.

P2350**Board Number: B1031****A synthetic yeast model for differentiation and division of labor.**M.E. Wahl¹, A.W. Murray^{1,2};¹Department of Molecular and Cellular Biology, Harvard University, Cambridge, MA, ²Harvard FAS Center for Systems Biology, Cambridge, MA

Somatic cells undergo permanent, heritable changes in gene expression that prevent them from replicating the complete organism. Despite the fitness costs entailed by this forfeit of reproductive

capacity, differentiation has evolved independently several dozen times, driving speculation on the compensatory fitness benefits and pre-adaptations necessary for the evolution and maintenance of differentiation. Due to the rarity and long timescale of these processes in nature, hypotheses generated through retrospective inference and modeling have awaited an experimentally-tractable biological model system in which they can be directly tested.

Borrowing the toolkit of synthetic biology, we engineer differentiation in the budding yeast *S. cerevisiae*. We implement inducible and tunable conversion and cell type-specific growth rates, irreversible differentiation through recombinase-mediated gene excision, terminal differentiation by removal of an essential cyclin-dependent kinase gene, and cell type-specific protein expression, including fluorescent markers. By combining this differentiation system with yeast mutations known to prevent cell separation following cytokinesis, we test a hypothesis concerning the order in which multicellularity and differentiation may evolve naturally. We also confirm an analytical result concerning the effect of spatial context on the maintenance of stem cell populations and the steady-state ratio between cell types. The strains developed here may further be used to study other major evolutionary transitions, including the appearance of body plans and life cycles, as well as mutation-selection balance.

P2351

Board Number: B1032

Paracrine rescued lobulogenesis in PR null mammary outgrowths by redirected testicular cells.

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Stem and progenitor cells in most adult tissues reside in specialized, highly regulated microenvironments called stem cell niches. We have previously shown that testicular, neural, bone marrow and cancer cells respond to the signals provided by the mammary niche (stroma, epithelial cells, local and extrinsic factors) and alter their cell fate to that of mammary epithelial progenitor cells. To determine if paracrine signals from progesterone receptor (PR) expressing mammary epithelial cells are required for this redirection, we mixed 100K PR null epithelial cells with 50K testicular cells from adult male mouse seminiferous tubules. The testicular cells were redirected *in vivo* to mammary epithelial cell fate during regeneration of the mammary epithelium and in the process rescued the developmentally deficient PR null cells, signaling them to produce alveolar secretory structures in the chimeric mammary outgrowths at parturition. We identified redirected testicular cells by staining with anti-PR and anti-RANKL antibodies. Positive cells were found both in ducts and in secretory structures. PR null outgrowths were uniformly negative for anti-PR staining. PR and RANKL positive testicular-derived cells were detected in second-generation outgrowths as well. This demonstrates that PR signaling is not required for cellular reprogramming of testicular cells to mammary epithelial cell fate *in vivo*. In addition, the testicular cells, which did not express PR prior to reprogramming, but were capable of providing the necessary paracrine signals required for lobular development of the PR null epithelium. These results demonstrate that once in the mammary niche, reprogrammed cells from other tissues can

not only receive signals from their new environment, but also contribute progeny that provide paracrine signals, which contribute to a “normalized” microenvironment.

Protist Cell Biology and Control of Parasites

P2352

Board Number: B1034

Plasmodium falciparum Double C2 Domain Protein, PfDOC2, Binds to Calcium when Associated with Membranes.

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The pathogenesis of malaria is strongly correlated with secretion of the micronemes, the apical organelles which contain the adhesins required for invasion of *Plasmodium falciparum* into human erythrocytes. A critical event in *P. falciparum* erythrocyte invasion is the production of calcium transients. After entering the cell, Ca²⁺ binds to soluble Ca²⁺-binding proteins, such as the double C2 domains (DOC2). Recently, deletion of a *P. falciparum* DOC2 protein, PfDOC2, was shown to cause impairment in microneme secretion. However, PfDOC2 remains poorly characterized. Here, we report that PfDOC2 is expressed throughout the erythrocytic cycle and demonstrate that it is associated with membrane fractions and binds to calcium when it is part of these membranous structures. In summary, we show that PfDOC2 is a calcium lipid-binding protein of the protein kinase C type of DOC2 proteins.

P2353

Board Number: B1035

Molecular characterization of apical membrane antigene-1 of *Eimeria tenella* isolates from Nigeria.

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The present study aimed at sequence characterization of apical membrane antigen 1 (AMA-1), a sub-unit vaccine candidate-encoding locus from Nigerian field *Eimeria tenella* isolates for comparison with reference strains from different parts of the world. The full length *E. tenella* AMA-1 (EtAMA-1) coding region was amplified by PCR from cDNA made from *Eimeria* oocyst samples obtained from three different commercial poultry farms in Nigeria. PCR fragments of interest were gel excised and purified using minelute gel purification, cloned using pGEM[®]-T Easy (Promega) vector system kits and transformed into XL1-Blue *Escherichia coli* (Stratagene). Candidate plasmids identified by EcoRI digestion and gel electrophoresis were sequenced (GATC Biotech) using the T7 and SP6 sequences within the T

Easy plasmid and analysed using CLC Main Workbench version 5.7.1 (CLC Bio, Denmark). Ten clones were assembled for each sample for comparison for possible polymorphism among the clones and with isolates from different parts of the world. Principal coordinate analysis results of the AMA-1 cDNA sequences and translated amino acid sequences indicated the occurrence of limited genetic diversity among EtAMA-1 alleles of field *E. tenella* isolates from within and outside the country, with minimal diversity compared to archived laboratory strains. This work therefore, highlighted the need to identify the major EtAMA-1 allelic types circulating in the population for inclusion into a possible multi-allele AMA-1 based anticoccidial vaccine, an approach that is proving to be promising in the development of effective malaria vaccines.

P2354

Board Number: B1036

Human antibody responses to surface antigens of *Plasmodium falciparum* gametocyte-infected erythrocytes and their relation with gametocytaemia.

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BACKGROUND: In *Plasmodium falciparum*, only mature gametocytes are found in peripheral circulation and available to uptake by the mosquito vector. Efficient transmission of gametocytes from the human host to mosquitoes contributes to the persistent burden posed by malaria. There is little evidence to suggest that natural immune responses to circulating gametocytes play a role in clearing gametocytes. **METHODS:** Here, magnet-purified and enriched mature gametocytes obtained from a laboratory parasite, 3D7, and a clinical isolate from Kenya, HL1204, were used to test for plasma antibody recognition to the surface of gametocyte-infected erythrocytes (GSA) and to measure longitudinal antibody responses in a cohort of Ghanaian school children using flow cytometry. **RESULTS:** Analysis of plasma antibodies in three sequential weekly samples from 113 asymptomatic *Plasmodium falciparum*-infected individuals showed that a proportion of the children exhibited marked antibody responses that recognized GSA on both 3D7 and HL1204. Some responsive individuals maintained their antibody levels during the study period, irrespective of concurrent gametocyte carriage status. Children with GSA antibodies present at enrolment, were less likely to develop new gametocytaemia at subsequent visits (odds ratio = 0.29, 95% CI 0.06 - 1.05; P = 0.034). **CONCLUSION:** Our data support the hypothesis that conserved antigens exist on the surface of gametocyte-infected erythrocytes in malaria parasites which elicit natural human antibody responses, and that these play a role in reducing gametocyte carriage. The identification of these novel gametocyte surface antigens and their evaluation as potential anti-gametocyte vaccine candidates and/or biomarkers of gametocytes are imminent tasks.

P2355**Board Number: B1037****Three dimensional structure of Maurer's cleft with tiny filaments in Plasmodium falciparum-infected Erythrocytes by "unroofing" method.**E.H. Hayakawa¹, F. Tokumasu², J. Usukura³, H. Matsuoka¹, T. Tsuboi⁴, T.E. Wellems⁵;¹Lab of Medical Zoology and Parasitology, Jichi Med University, Shimotsuke, Japan, ²Department of Lipidomics, The university of Tokyo, Tokyo, MD, ³Structural Biology Center, Nagoya University, Nagoya, Japan, ⁴Cell-Free Science and Technology Research Center, Ehime University, Matsuyama, Japan, ⁵Lab of Malaria and Vector Research, NIAID/NIH, Bethesda, MD

Plasmodium falciparum, human malaria parasites, delivers over 400 proteins to the surface of erythrocytes has no protein transport machinery. Maurer's clefts (MCs) appear in parasitized erythrocyte cytoplasm after invasion and are believed to play a crucial role for protein transportation. Recent data with 3-D reconstruction of thin-layer transmission electron microscopy (TEM) estimated Maurer's cleft structure, but much details are still unknown due to the loss of fine structure information while thin-layer preparation. In this study, we used "unroofing/rip-off" technique and investigated erythrocyte membrane skeleton and MCs from cytoplasm side by TEM. We found oval/global structure connected through fine extensions consistent with estimated MC characteristics. These extensions have 170 – 450 nm in length and

P2356**Board Number: B1038****Cotranscription of Wolbachia surface protein (WSP) with Type IV Secretion System (T4SS) in Wolbachia pipientis wStr.**Y. Li¹;¹Entomology, University of Minnesota, St Paul, MN

Wolbachia is a cytoplasmically transmitted alpha-proteobacterium and is able to induce several reproductive alternations in its arthropod hosts, including cytoplasmic incompatibility (CI), feminization of the males, male killing and parthenogenesis. *Wolbachia* surface protein (WSP) has a transmembrane structure and its participation in host immune response and apoptosis inhibition makes it important for bacterial survival and proliferation within the host. *Wsp* gene is downstream of *virD4* gene that belongs to one of the two Type IV Secretion System (T4SS) operons. T4SS is a membrane spanning apparatus that has 12 components (VirB1-11 and VirD4), most of which are translated from two cotranscribed gene clusters (operon *virB8-11*, *virD4* and operon *virB3-6*). The objective of this study is to analyze the cotranscription of *wsp* and *virD4* genes in *wStr*, a *Wolbachia pipientis* strain that maintains a robust infection in an *Aedes albopictus* mosquito cell line. Total RNA was purified from infected and uninfected control cells. After purified RNAs were verified by polymerase chain reaction (PCR) for absence of genomic DNA contamination, reverse transcriptase (RT)-PCR was used with a pair of primers that

overlaps both *virD4* and *wsp* genes, and the resulting cDNA amplicon was sequenced. Results showed that the cDNA amplicon obtained had the expected size (528bp) after electrophoresed on a 1% agarose gel, and its nucleotide sequence verified the overlap of both *virD4* and *wsp* genes. These results indicate that *wsp* gene is cotranscribed with *virD4* and the upstream T4SS genes. It is widely believed that *Wolbachia* manipulate host reproductive alternations through T4SS-mediated secretion of effector proteins into host reproductive tissues. The fact that *wsp* is cotranscribed with T4SS genes suggests its possible interaction with T4SS and potential role as an effector protein. *Wsp* gene has an extensive genetic divergence in nature, however searching for the precise role it plays in the interaction between *Wolbachia* and host needs future efforts.

P2357

Board Number: B1039

Differential proliferation of *Wolbachia* endosymbiont in *Aedes* mosquito cell line.

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The cell lines of *Aedes albopictus* (C636) obtained from NIMHANS, Karnataka, India were maintained in incubators with M&M insect cell culture medium and BSA to establish *Wolbachia* an intracellular proteobacteria. The cell lines were inoculated with *Wolbachia* (A, B and AB super groups) and the strength of the infection was calculated based on the perpetuation of the cells in the in-vitro medium. The comparison between control and infected cell lines suggests a two-fold decrease in infected cells reaching confluence. The inoculum strength differs from the source of *Wolbachia* isolated from different insect hosts. *Wolbachia* extracts with double infections (AB) are highly virulent than single infections. Among single infections, *Wolbachia* B supergroup is more virulent than A supergroup. Further, it was observed that *Wolbachia* derived from *Exorista sorbillans*, *Aedes albopictus*, *Trichogramma japonicum* has greater virulence and cell lines can be infected within few passages. *Wolbachia* isolated from *Talicauda nyseus* did not induce any significant effect on mosquito cell lines. The findings of the current study append the database of potential non-native *Wolbachia* strains that can be introduced in mosquitoes for expression of novel phenotypes. Recent findings reports increased virulence of pathogenic West Nile Virus, when confounded with native *Wolbachia* strains in mosquitoes. Thus screening of alternative *Wolbachia* strains that could be maintained in mosquito cell lines and establishing a 'Wolbachia strain pool' for mosquito trans-infection is of significant importance.

Keywords : *Aedes albopictus*; cell lines; *Wolbachia* infections; virulence

P2358**Board Number: B1040****High molecular weight protein associated to the flagellar attachment zone in *Trypanosoma brucei* cytoskeleton.**M.M. Baqui¹, B.P. Moreira¹;¹Cell and Molecular Biology, University of Sao Paulo, Ribeirao Preto, Brazil

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Trypanosoma brucei is a pathogenic unicellular eukaryote that infects humans and other mammals in sub-Saharan Africa. The causative agent of sleeping sickness, *Trypanosoma brucei*, represents an excellent model for cell biology suitable for investigation analysis of basic questions about the cytoskeleton and its evolution in higher eukaryotes. The most prominent organelle, the flagellum harbors most of the cytoskeletal content of the cell and is responsible for cell division, motility, morphogenesis and infectivity. It comprises the axoneme and paraflagellar rod and both are connected to the cell body via the flagellar attachment zone (FAZ). In all genera of the Trypanosomatidae family, we described a novel class of high molecular mass phosphoproteins associated to the cytoskeleton, which, besides their structural function, might play a role in the organization and regulation of cytoskeleton and its constituents. Cytoskeleton of *T. brucei* was obtained after treating parasites with lysis buffer containing TX-100 detergent following its solubilization with 1M NaCl to isolate flagella from the cell body skeleton. SDS-PAGE gradient gel and silver staining revealed the distribution of the high molecular weight protein in all extracts analysed but most concentrated in the flagellar fraction. In order to generate a polyclonal serum, giant protein from the flagellar fraction was cut out from the gel and injected in Balb/C mice. Confocal immunofluorescence microscopy and immunoelectron microscopy revealed the localization at a specific region between the cell body and the flagellum corresponding to the flagellar attachment zone (FAZ). Moreover, this protein did not co-localize with tubulin (axoneme) neither with paraflagellar rod (PFR) suggesting its participation in the FAZ structure. The corresponding giant protein band was excised from the gel and sent to mass spectrometry (MS) analysis. We retrieved a list with putative candidates most of them with no function described so far. Known candidates included calpain-like protein, which has been shown to be essential to remodel parasite shape. The next steps include the expression of tagged proteins and an RNAi assay to reveal what role they might play in the cell.

P2359**Board Number: B1041****Characterization of Inositol Phosphate Pathway in *Trypanosoma brucei*.**C. Cordeiro¹, A. Saiardi², R. Docampo³;

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Trypanosoma brucei is a protozoan parasite that infects people and animals. It causes human sleeping sickness, a deadly illness that lead to death if untreated. *T. brucei* is prevalent in sub-Saharan Africa, where it infects thousands of people and poses treat to millions. The lack of vaccines and limited treatment options underlines the need for research on new drug targets. We are studying the inositol phosphates signaling pathway of trypanosomes. Inositol phosphates are polyphosphorylated myo-inositol molecules that play various roles in different cells. Genes coding for kinases that synthesize inositol phosphates are found in all eukaryotes. We identified three kinases that belong to this pathway in *Trypanosoma brucei* genome: Inositol Polyphosphate Multikinase (TbIPMK), Inositol Pentakisphosphate 2-kinase (TbIPPK) and Inositol Hexakisphosphate Kinase (TbIP6K). We used in vitro activity assays to determine substrate specificity and reaction products of the three enzymes. TbIPMK phosphorylates IP3 to synthesize IP5 and PP-IP4. TbIPPK synthesize IP6 using IP5 as substrate. Finally, TbIP6K phosphorylates IP5 and IP6 to form PP-IP4 and IP7, respectively. We also characterized the kinetic parameters of each kinase at different concentrations of ATP and substrate. Complementation of mutant yeasts demonstrated the ability of TbIPMK, TbIPPK and TbIP6K to synthesize inositol phosphates in living cells. Yeast mutants depleted of the IPMK and IP6K homologs have slow growth rate. TbIPMK and TbIP6K complementation in yeast restored normal growth and wild type inositol phosphate levels. We also analyzed *T. brucei* extracts for the presence of inositol phosphates using polyacrylamide gel electrophoresis and high performance liquid chromatography. Interestingly, we could only detect IP, IP2 and IP3 in *T. brucei* procyclic (insect stage) and bloodstream forms (mammalian stage). The highly phosphorylated inositol phosphates were not detected in extracts from cultured parasites, although they express the active enzymes capable of synthesizing them. We are currently investigating functions of the inositol phosphate pathway for *T. brucei* survival upon different stress conditions. Our long-term goal is to understand the functions of this pathway in trypanosomes. The study of inositol phosphates in trypanosomes may lead to better understanding of these molecules in other related organisms and provide new targets for drug discovery.

P2360**Board Number: B1042****Molecular Tools to Probe Signaling Events During Single Cell Regeneration in the Giant Ciliate Stentor.**S. Reiff¹, M.M. Slabodnick¹, P. Sood¹, T. Makushok², G. Ruby¹, J. DeRisi¹, W. Marshall²;¹University of California, San Francisco, San Francisco, CA, ²Biochemistry and Biophysics Dept., University of California, San Francisco, San Francisco, CA

The unicellular ciliates of the genus *Stentor* have the unique ability to fully regenerate after being cut in half, in a way that perfectly preserves cell polarity and structure. There are potentially many parallels between the mechanisms underlying this process and those involved in development and differentiation. Although regeneration in *Stentor* used to be a popular area of study for microscopists, so far the molecular details behind this incredible phenomenon have remained unstudied, in part because of the lack of genetic tools. Recently, however, our laboratory has initiated sequencing of the *Stentor coeruleus* genome, and has developed a system for RNAi knockdown of *Stentor* genes. To gain a better understanding of the regeneration process, with the help of Radiant Genomics we have developed constructs for RNAi knockdown of 1300+ different genes. This large selection of genes consists of a wide variety of protein families. A few of these categories include genes for signaling components such as kinases, GPCRs, calcium channels, and guanylyl cyclases, and proteasomal components. RNAi screening of these individual genes is ongoing, and will ultimately reveal some of the signaling pathways that coordinate the many different precisely timed cellular events required for successful regeneration. Concurrently, a system for tagged transgene expression in *Stentor* is also being developed to facilitate downstream characterization of important proteins identified by this screen. An expression plasmid based on the *Stentor* calmodulin gene has been constructed, containing either a C-terminal GFP or triple HA tag. Experiments are now underway to reveal which electroporation conditions are able to most efficiently deliver plasmid DNA to the nucleus. In the future, a better understanding of the mechanisms behind single cell regeneration will have important implications for basic biology as a whole, and will inform the most fundamental questions in cell biology about how single cells can establish and maintain their polarity, morphology, and size.

P2361**Board Number: B1043****The genetic and physical basis of diatom cell wall patterning.**J.J. Russell^{1,2}, J.A. Theriot^{2,3};¹Department of Biology, Stanford University, Stanford, CA, ²Department of Biochemistry, Stanford University School of Medicine, Stanford, CA, ³Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA

Diatoms are photosynthetic microbes that precipitate silica to form intricately structured glass cell walls, termed frustules. Frustules exhibit species-specific morphologies, including highly reproducible nano- to

micro-scale features. My project aims to develop Crispr-Cas9 genome editing in two sequenced diatoms, *Phaeodactylum tricornutum* and *Thalassiosira pseudonana*, to facilitate a genetic approach to understanding frustule patterning. A genome-wide forward genetic screen for frustule defects aims to identify genes responsible for large- and small-scale frustule morphology. Clonal populations of *P. tricornutum* exhibit three distinct morphotypes, the regulatory genes for which may be identified in mutant populations lacking one or more morphotypes. Mutants with altered nano- to micro-scale features will also be genotyped and characterized. Next, a reverse genetic approach applied to several genes known to encode frustule-localized proteins will test how specific sequence motifs specify morphologic characters. Finally, genes from a diversity of diatoms will be heterologously expressed to determine the potential for engineering chimeric frustules.

P2362

Board Number: B1044

Factors that Influence the Life Cycle of a Marine Ciliate Co-Isolated with Eggs of the Sea Urchin *Lytechinus Variegatus*.

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This study focused on determining environmental factors that trigger the encystment of a ciliated marine protist that was originally isolated as a contaminant of harvested eggs from the green sea urchin *Lytechinus variegatus*. Upon induced spawning of female urchins, eggs appear to be protist free. Within 24 hours, protists begin to appear and feed on disintegrating eggs. Replicating forms of the protist quickly multiply to high numbers. The vegetative form of the ciliate was recovered and cultures have been maintained at room temperature in media composed of artificially prepared sea water supplemented with a commercial fish food, Marine-S. Experiments were performed in which these protists were grown in culture under conditions of varying temperature and food supply. Cultures were monitored by microscopy at 25 °C and at 4 °C for several days. Results indicated that at room temperature, the vegetative form of the ciliate reproduced exponentially, reaching a density of 10⁵ protists/mL by the fourth day. By the fifth day, however, protist numbers began to decrease rapidly in proportion to an increase in the number of cysts observed. When separate cultures were inoculated with the vegetative protist and placed at 4 °C, only cysts could be seen after 24 hours. The results suggest that this protist becomes encysted in response to environmental stresses of decreased temperature and food depletion. By preliminary analysis, the isolated protist appears to be related to the species *Orchitophrya stellarum*, a parasitic ciliate that resides in the testes of male starfish. Further molecular studies will be done to confirm identification. Whether a similar symbiotic relationship exists between female *L. variegatus* sea urchins and an encysted form of this isolated protist will also be investigated. Possible roles for this organism in marine ecosystem structure will be discussed.

P2363**Board Number: B1045****Identification of two protozoan contaminants of primary cell cultures from the sea star, *Patiria miniata*.**G. Reyes¹, Y. Lee¹, F. Hajredini¹, J. Khan¹, A. Manassero¹, E. Quezada¹, I. Macebuh¹, F. Abreu¹, S. Ahmed¹, A. Tehuitzil¹, H. Alia¹, S. Singh¹, D. Fugar¹, T. Onorato¹;¹Natural Sciences, LaGuardia Community College, Long Island City, NY

During the Spring 2014 semester at LaGuardia Community College, students in the Cell Biology capstone course cultured cells isolated from various tissues of the sea star, *Patiria miniata*. Despite the addition of an antibiotic-antimycotic solution containing penicillin, streptomycin and Fungizone® (amphotericin B), primary cell cultures were contaminated by two unicellular protists. Since microbial contamination is a major obstacle in culturing marine invertebrate cells, we decided to determine the identity of these two protists by using cell biology techniques and metagenomics. Cells were imaged using an EVOS® FLoid® Cell Imaging Station from Life Technologies. The large protist appeared to be a ciliate while the small protist to be a flagellate, as indicated by light microscopy. Staining the DNA of live cells with NucBlue® Live ReadyProbes® reagent revealed the presence of macro- and micronuclei, further confirming that the large protist was a ciliate. No macronuclei were seen in the small protist and its movement was characteristic of an euglenoid. Additionally, lipid membranes of live cells were visualized with the fluorescent stain, DilC12(3) perchlorate. Numerous lipophilic vacuoles were present in the ciliate. Subsequently, metagenomic analyses were performed to identify these protists at the molecular level. Genomic DNA was isolated and sent to Molecular Research LP (Shallowater, TX) for 18S rRNA analysis. The 18S rRNA biodiversity assay revealed that the flagellate belongs to the order *Diplonemida* and the ciliate was identified as *Paranophrys marina*. These metagenomic data confirmed our microscopic observations. Identification and further characterization of these protozoan contaminants will not only help advance the field of marine invertebrate cell culture but may also aid in elucidating the causative agent(s) of the sea star wasting disease. (Funded in part by grant 5T36GM101995-01)

P2364**Board Number: B1046****Release of malaria parasites from red blood cells requires parasite-derived pore-forming proteins.**

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Malaria is a disease caused by asexual replication of Apicomplexan parasite *Plasmodium falciparum* inside human erythrocytes. However, parasites must shuttle between humans and mosquitoes to complete its complex life cycle. Human-to-human transmission of infection happens through the bite of infected mosquitoes, where the parasite sexual stage takes place. Both the end of the asexual cycle in human erythrocytes and gamete fertilization in mosquitoes requires egress of parasites from the host erythrocytes. Here we show that erythrocyte membrane perforation precedes egress of sexual and asexual forms of parasites from infected erythrocytes. Using a novel approach and applying fluorescent live cell microscopy we demonstrated that membrane perforation happens a few seconds prior to asexual parasite egress and a few minutes before the emergence of sexual forms from erythrocytes. The pores are large enough for diffusion of hemoglobin and GFP-tagged parasite-derived proteins from the erythrocyte cytoplasm before erythrocyte membrane rupture and parasite egress. Erythrocyte swelling apparently is not needed for successful membrane perforation. Positive-curvature amphiphiles promote parasite egress, and membrane sealants block the process. *Plasmodium falciparum* encodes five perforin-like proteins (PPLP1-5). There is no direct data indicating which is involved in the asexual parasite cycle. Here we show that PPLP2(-) parasites have a normal asexual cycle but are unable to complete the sexual stage of the life cycle. Activated sexual forms remain trapped within erythrocytes whose membranes are impermeable for hemoglobin and fluorescent probes. The physiological consequence of PPLP2 deletion is the inability of malaria parasites to transfer infection from human to mosquito. Thus, the perforation of the erythrocyte membrane is an essential step in parasite egress and parasite life cycle continuation.

P2365**Board Number: B1047****Calcium Dynamics In The Human Malaria Parasite Plasmodium Falciparum Detected By Genetically Encoded Ca²⁺ Indicator.**L. Borges-Pereira¹, B.R. Koresch Leiva Campos², C.R. Garcia³;¹Departament of Parasitology, University of Sao Paulo, Sao Paulo, Brazil, ²Departament of Physiology, University of Sao Paulo, Sao Paulo, Brazil, ³Departament Physiology, University of Sao Paulo, Sao Paulo, Brazil

Calcium (Ca²⁺) is a ubiquitous signaling molecule in all eukaryotic cells, modulating several physiological processes. Changes in Ca²⁺ concentration regulate muscle contraction, neurotransmitter secretion and metabolic pathways. In the life cycle of *Plasmodium falciparum*, the etiological agent of human malaria, Ca²⁺ is involved in motility, cell progression and cell invasion and egress from red blood cells (RBCs). We developed a transgenic line of *P. falciparum* expressing GCaMP3, a genetically encoded calcium indicator (GECI). The ORF encoding the GCaMP3 gene was cloned in the transfection plasmid pDC and used to transfect a synchronized ring culture of *P. falciparum*. The transfected population was submitted to a cell sorting by flow cytometry to select those parasites with increased fluorescence. One clone was selected and the calcium response was tested in the presence of the calcium ionophore ionomycin and the SERCA (sarco / endoplasmic reticulum Ca²⁺ ATPase) inhibitor thapsigargin. Our results demonstrate that PfGCaMP3 parasites are responsive to Ca²⁺ alterations, showing an increased fluorescence when exposed to compounds that cause Ca²⁺ release in the cytoplasm. This novel transgenic parasite will provide new insight on calcium homeostasis, allowing the screening and identification of new classes of compounds with anti-malarial activity capable of interfering with signaling pathways controlling parasite growth and development.

P2366**Board Number: B1048****Lb_0210 has an osmotic effect in Leishmania, essential for differentiation and replication in macrophages.**

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Introduction: Leishmania transits in its life cycle between environments that differ in pH, and osmolarity. Securing transport of nutrients requires H⁺-ATPase pumps that generate an electrochemical gradient to serve this coupled to anion currents. Inhibition of these ion currents alters the capacity of the parasite to control pH and cell volume. In *Leishmania (V) braziliensis*, four sequences are putative CLC chloride channels. The aim of this study was to determine the role of the protein codified by one of them: LBRM010210. Materials and methods: From pGEMT-EasyLb0210 the restriction sites *Bam*HI and *Hind*III were inserted by PCR and the product ligated into p416Met25 before digestion and ligation into pSGEM that was used as a template to synthesize cRNA with the mMESSAGING mMACHINE® kit. To determine voltage and pH dependency *Xenopus laevis* oocytes were injected with 50 ng Lb0210 cRNA. Oocytes were recorded with voltage clamp in the presence of different concentrations of Cl⁻ or pH. Special primers were designed to induce interference by hairpin structures. A conventional PCR was carried out including the restriction sites *Xba*I and *Eco*RI and the fragments then digested and self-ligated before subcloning into pSP72RaneoαGFP. *L. (V.) braziliensis* promastigotes were electroporated with the interfering construct and selected with 60 μg/ml G418 in Schneider's medium plus 20% FBS. The fluorescent parasites were followed in culture, osmotically challenged and used to infect the macrophage-like cell line J774A.1. Parasite growth, shape and movement were determined. Solutions with different osmolarities were used and parasite volume determined by impedance using Beckman Coulter z2®. Infection was followed by light microscopy and percentage of infection, parasite number, parasite transformation and parasitophorous vacuole (PV) volume determined. With analysis of variance statistical differences were accessed and when found the Bonferroni post-hoc test was used (SigmaStat 3.5). Results: Microinjection of Lb0210 cRNA in *X. laevis* oocytes, induced voltage dependent outward rectifying currents of low amplitude. By 72 hours post-injection Lb0210 oocytes had also increased fragility and vitelline membrane detachment indicating volume changes. Transformation of promastigotes with the interfering construct render viable, fluorescent parasites that had greater volume, different swimming pattern, and showed impaired volume regulation compared to wild type, particularly at osmolarities lower than 70 mOsm/L. They were also unable to differentiate into amastigotes or replicate within the PV after 96 h post-infection in J774A1 macrophages. Conclusion: Lb_0210 appears to be important in volume and osmotic regulation and amastigote differentiation.

Prokaryotic Cell Biology

P2367

Board Number: B1049

Complex two-component regulation of bacterial surface adhesion via integrated transcriptional control of *hfiA*, a potent adhesion inhibitor.

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In natural environments, bacteria often adhere to surfaces where they form complex multicellular communities. Surface adherence is determined by the biochemical composition of the cell envelope. *Caulobacter crescentus* secretes a polar adhesion called a holdfast, which enables permanent attachment to surfaces. *Caulobacter* employs a novel regulatory mechanism that integrates cell cycle, nutritional and environmental signals to control development of this adhesive envelope structure. Specifically, we have discovered a 68-residue protein inhibitor of holdfast development (HfiA) that directly targets a conserved glycolipid glycosyltransferase required for holdfast synthesis (HfsJ). Our data suggest that post-translational regulation of cell envelope enzymes by small proteins like HfiA may provide a general means to modulate the surface properties of bacterial cells.

The promoter of *hfiA* is affected by both numerous signaling inputs to ensure appropriate control of surface adhesion. Multiple cell cycle regulators directly control *hfiA* expression and temporally constrain holdfast development to the late stages of G1. HfiA further functions as part of a 'nutritional override' system that decouples holdfast development from the cell cycle in response to nutritional cues. While the mechanism of nutritional sensing is unknown, this control feature can limit surface adhesion in nutritionally sub-optimal environments without affecting cell cycle progression. Moreover, we have shown that the photosensory two-component system, LovK-LovR, regulates *hfiA* expression and holdfast development. However, the response regulator LovR lacks a DNA-binding output domain and thus the mechanism of transcriptional regulation must be indirect.

To better understand how the LovK-LovR environmental sensory system regulates *hfiA* expression, we designed a forward genetic selection to identify mutants in which transcriptional regulation of *hfiA* by LovK-LovR is disrupted. In this screen, the *hfiA* promoter controls the expression of chloramphenicol acetyl transferase (CAT), and growth in the presence of chloramphenicol allows for selection of mutants with increased *hfiA* promoter activity. Selected mutants were further evaluated to confirm faster growth in chloramphenicol, regulation of a second promoter fusion (*PhfiA-lacZ*), and regulation of adhesion. Using whole genome sequencing, we identified mutations in additional two-component signaling genes: a LOV interacting hybrid histidine kinase (LihA), histidine kinase (LihA), and response

regulator (LirA). Our preliminary analyses suggest that direct interactions between LovK, LihA, LikA regulate the *hfiA* promoter via the DNA binding domain of LirA.

P2368

Board Number: B1050

Quantitative analysis of E-cadherin-mediated *Listeria monocytogenes* invasion of epithelial cells.

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The Gram-positive pathogenic bacterium *L. monocytogenes* invades a wide variety of phagocytic and non-phagocytic host cells. For invasion of epithelial cells, the bacterial surface protein Internalin A (InIA) binds to host cell E-cadherin, which induces local actin polymerization, remodels the host's membrane topology, and culminates in bacterial uptake via clathrin-mediated endocytosis. Experiments with E-cadherin truncation mutants have suggested that the ectodomain is sufficient for *L. monocytogenes* adhesion to the cell surface but interaction of the cytoplasmic domain with beta-catenin is necessary for invasion, and phosphorylation of E-cadherin profoundly regulates its affinity for beta-catenin. We have used epithelial cells expressing non-phosphorylatable and phosphomimetic point mutations in the beta-catenin-binding domain of E-cadherin to determine the quantitative effects of these modifications on bacterial adhesion and invasion. The non-phosphorylatable mutation results in lower surface expression of E-cadherin, but surprisingly neither this nor the phosphomimetic mutation affects the amount of InIA-dependent *L. monocytogenes* adhesion or invasion. Expression of InIA in the non-pathogenic *L. innocua* is sufficient to confer normal levels of both adhesion and invasion and is comparable for wild type, phosphomimetic, and non-phosphorylatable E-cadherin. Furthermore, we find that bacterial expression of Internalin B (InIB), which activates the host cell growth factor receptor c-Met, is not required for normal adhesion or invasion. We conclude that the efficiency of InIA/E-cadherin mediated adhesion and invasion is likely to be a non-linear function of E-cadherin concentration. As our next steps, we propose experiments to: (1) quantitatively analyze how E-cadherin levels regulate *L. monocytogenes* invasion, and (2) study the pathogenesis of listeriosis in a more physiologically relevant context using intestinal organoids.

P2369**Board Number: B1051****Structure of the CTP synthetase filament: a novel cytoskeletal element.**

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A growing number of metabolic enzymes have been shown to form filaments in cells, constituting novel cytoskeletal systems. Although the functional significance of enzyme polymerization is unclear in most cases, increasing evidence suggests a link between filament assembly and regulation of enzymatic activity. Here, we report the first high-resolution molecular structure of the filamentous form of a metabolic enzyme, CTP synthetase (CtpS), providing insight into the mechanism and functional consequences of polymerization. CtpS is essential for cell proliferation, and was recently shown to form filaments in both prokaryotes and eukaryotes, suggesting deep evolutionary conservation of CtpS polymerization. We show that filament assembly plays a critical role in inhibiting CtpS enzymatic activity, likely by sterically locking enzyme subunits into an inactive conformation. Structure-guided mutagenesis indicates that linking activity to polymerization promotes cooperative catalytic regulation. This previously uncharacterized regulatory mechanism is important for cell function since a CtpS mutant that is enzymatically active but unable to polymerize disrupts *E. coli* growth and metabolism. We propose that coupling filament assembly to enzyme activity enables ultrasensitive control of activity while storing inactive enzymes in a readily accessible form; this may represent a general mechanism of regulation in other filamentous metabolic enzymes.

P2370**Board Number: B1052****Elucidating the structure and function of the *Vibrio parahaemolyticus* T3SS1 effector VopR.**

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The gram negative γ -proteobacteria, *Vibrio parahaemolyticus* (*V. para*) is a food-borne pathogen that naturally inhabits marine and estuarine environments. *V. para* is one of the most ecologically versatile of the *Vibrio* species due, in part, to its expanded genome and has been marked as an emerging pathogen and a leading cause of acute seafood-borne gastroenteritis worldwide. As part of its repertoire of virulence factors, the *V. para* genome encodes two type III secretion systems (T3SS). T3SSs are macromolecular machines derived from the bacterial flagellar apparatus that efficiently translocate bacterial effector proteins directly into the cytoplasm of host cells. The first system, T3SS1, which is anciently acquired and found in all *V. para* isolates, orchestrates a regimented host cell infection by

inducing autophagy, cell rounding and finally cell lysis. Several T3SS1 effectors, including *VopR*, remain uncharacterized. VopR is a 325 amino acid protein that has been found to induce HeLa cell rounding and inhibit yeast growth, as well as localize to the host cell membrane through binding P(4,5)IP₂ via a N-terminal phosphoinositol binding domain (BPD). This BPD is conserved in T3SS effector proteins of many mammalian and plant pathogens and is likely very important for effector folding and localization within the host cell. The host cellular activities targeted by VopR are unknown, as the catalytic activity and molecular targets of VopR within host cells remain undefined. Here, we seek to identify the host cell targets and to determine the catalytic function of VopR. In addition, we are employing structural studies to gain a better understanding of both the conserved N-terminal BPD and C-terminal catalytic domain of VopR. Using bioinformatics we have identified the putative C-terminal catalytic domain of VopR. We also discovered that of both the N-terminal BPD and the C-terminal catalytic domain of VopR contribute to toxicity in yeast and HeLa cell rounding, suggesting that VopR acts on its targets specifically at the host cell membrane. A yeast two hybrid against a mouse cDNA library revealed several putative targets of VopR, which we are currently testing in both *in vivo* and *in vitro* studies.

P2371

Board Number: B1053

The N-terminal domain of MinE exhibits amyloidogenic property that underlies formation of the MinE ring.

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Pole-to-pole oscillation of the Min proteins regulates the cell division site placement in *Escherichia coli*. MinE of the Min system organizes into a ring-like structure that plays a critical role in triggering the oscillation cycle. Despite of its functional importance, the mechanism underlying the formation of the MinE ring remains unclear. Recently, we reported that MinE self-assembles into fibrillar structures on the supported lipid bilayer. Here, we present evidence that N-terminal domain of MinE possesses the amyloidogenic property via the Thioflavin T fluorescence and Congo red spectroscopic assays, far-UV circular dichroism measurements, and electron microscopy. The amyloidogenic sequence within the N-terminal domain of MinE is confirmed by proteinase K digestion and acid denaturation of the fibrils, followed by HPLC purification and N-terminal sequencing. In addition, the molecular dynamic simulation is used to build a model of the amyloidogenic region of MinE that demonstrates a characteristic cross- β structure of amyloid fibrils. Taken together, the results suggest that the amyloidogenic property of MinE may implicate in the mechanism of MinE-ring formation. Further studies will be performed to address the kinetic property of the ring-like structure in the form of amyloid aggregates during oscillation.

P2372**Board Number: B1054****Elucidating mechanisms of bacterial cell growth through live single-cell imaging and Bayesian model selection.**Z. Barry¹, E.C. Garner², M. Bathe¹;¹MIT, Cambridge, MA, ²Harvard University, Boston, MA

The viability of bacteria relies on the structural integrity of the peptidoglycan cell wall. A detailed understanding of the cell wall synthesis machinery is essential to enabling the discovery of new molecular targets for antibiotics due to the increasing emergence of bacterial resistance to existing compounds. The molecular machinery that synthesizes and maintains the cell wall structure consists of dozens of molecular components with complex and dynamic interactions that can only be properly resolved in the context of intact living cells. Single molecule imaging and computational analysis of the peptidoglycan synthesizing proteins (PGSP) offers the unique opportunity to mechanistically characterize the spatial activity, molecular interactions, and regulation of proteins that drive bacterial growth to elucidate important new antibiotic targets.

Toward this end, we have developed an experimental-computational approach that combines systematic fluorescence labeling and high-resolution imaging with targeted chemical and genetic perturbations. Single-molecule imaging of the proteins that coordinate peptidoglycan (PG) synthesis offers novel single-molecule insight into the process of cell wall synthesis and elongation. For example, it has been shown in rod-like bacteria that the PGSP protein MreB moves in a directed, circumferential manner around the short axis of the cell, possibly driven by the polymerization of new PG.

To capture cell elongation dynamics in real time, fluorescence movies of genetically engineered PGSP fusions in live, single cells are captured, and localizations of the molecules as they move throughout the cell are measured using single-particle tracking. Importantly, automated and objective Bayesian multiple hypothesis testing of competing molecular motion models is performed to elucidate molecular dynamics including binding lifetimes, protein-protein interactions, as well as their modes of motion on the outer cell membrane.

[1] Monnier, N., Guo, S. M., Mori, M., He, J., Lénárt, P., & Bathe, M. (2012). Bayesian Approach to MSD-Based Analysis of Particle Motion in Live Cells. *Biophysical Journal*, 103(3), 616-626.

[2] Garner, E. C., Bernard, R., Wang, W., Zhuang, X., Rudner, D. Z., & Mitchison, T. (2011). Coupled, circumferential motions of the cell wall synthesis machinery and MreB filaments in *B. subtilis*. *Science*, 333(6039), 222-225.

[3] Berglund, A. J. (2010). Statistics of camera-based single-particle tracking. *Physical Review E*, 82(1), 011917.

P2374**Board Number: B1056****Alternative Nucleic Acid Structures in the Macronucleus and Micronucleus of the Blepharisma.**A.E. Skiadas¹, D. Carbone¹, C. Gagna^{1,2,3}, I. Isquith⁴, N. Agostino⁴, A. Bradshaw⁴, W. Lambert^{2,3}, A. Khan¹;¹Department of Life Sciences, New York Institute of Technology, Old Westbury, NY, ²Department of Pathology and Laboratory Medicine, Rutgers-New Jersey Medical School, Newark, NJ, ³Department of Dermatology (Medicine), Rutgers-New Jersey Medical School, Newark, NJ, ⁴Department of Biology, Fairleigh Dickinson University, School of Natural Sciences, Teaneck, NJ

Blepharisma is a microphagus, filter feeding, light-sensitive ciliated protozoan, usually found in fresh water. Ciliates are considered the most complex and evolved of the protozoa. Blepharisma contains approximately 40 species with numerous strains and sub-varieties. *B. stoltei* is about 190 micrometers in length and deals with hazardous conditions by forming a cyst. It contains a large macronucleus and several micronuclei which are involved with sophisticated molecular biological processes, such as reproduction and regeneration. Nucleic acids are dynamic molecules that can adopt many different structures, such as right-handed double-stranded (ds-) B-DNA, single-stranded (ss-) DNA, left-handed ds-Z-DNA, triplex DNA and quadruplex DNA. The nuclei of the Blepharisma were immunohistochemically and immunochemically (ELISA) examined for the presence of alternative DNA structures. A variety of anti-ds-B-DNA antibodies, anti-ss-DNA antibodies, anti-Z-DNA antibodies and anti-methylated DNA antibodies were used. Additionally, we used the Feulgen reaction for determination of DNA content. We also examined these organisms for the presence of methylated DNA. Several different fixatives were used to preserve the organism's morphology and genome (i.e., preservation of intact, unaltered DNA), namely, 10% formalin, zinc formalin, Bouin's solution, Zenker's solution, Histochoice, Methacarn, Carnoy's solution, glutaraldehyde, and Davidson's fixative. This was done in order to determine morphology and the distribution/content of nucleic acids in the nuclei of the Blepharisma. Our data show that Blepharisma has all of these different types of DNA. However, the presence of left-handed Z-DNA was better demonstrated using Carnoy's solution. Bouin's fixative did not preserve DNA structure. Antigen retrieval was needed for some of these fixatives. Digestion of nuclear DNA with nicking concentrations of DNase I results in no Z-DNA immunoreactivity. Full nuclease digestion nicking concentrations resulted in no B-DNA immunoreactivity. Certain fixatives resulted in better preservation of morphology and others for certain DNA structures. Our data reveals that Blepharisma contains conventional and alternative nucleic acids. Z-DNA immunoreactivity depends on certain nuclear regions, physiological cycles and fixatives. Less Z-DNA immunoreactivity has been detected in non-cyst conditions. This type of research may shed light on the evolution of DNA structure and function. This research project was supported in part by a NYIT 2011 Institutional Support of Research and Creativity grant.

P2375**Board Number: B1057****Identification and Characterization of Plasmids from Marine Bacteria Isolated from the Sea Urchin *Lytechinus variegatus*.**D.A. Ballard¹, J. Chen², C. Priano¹, L. Jayant¹;¹Science, Borough of Manhattan Community College, New York, NY, ²SUNY at StonyBrook, Brooklyn, NY

Bacterial flora that coexist on the surface and test of the sea urchin *Lytechinus variegatus* were screened for the presence of cryptic plasmids. Several bacterial strains were isolated from the test, spines, mouth and gonads of urchins by gently swabbing these regions with a sterile tip and plating them onto marine agar plates. Strains were also isolated from the water samples in which the urchins arrived. Gram staining of more than sixty isolated bacteria revealed that all were gram negative rods. Isolated bacteria were tested for the presence of plasmids using a Bio Rad Aurum plasmid mini kit. Results indicated that at least two out of the sixty bacterial samples contained plasmid DNA. One of these bacterial isolates was taken from a spine that had been shed from an urchin. A red area at the base of the spine was used for the initial streaking. The second bacterial isolate was obtained from the water sample in which the urchins were shipped. Both plasmids are small, about 2.0 to 3.0 kb in size. 16S rRNA gene sequencing will be performed to further identify and characterize the bacteria that host these plasmids. Restriction mapping and DNA sequence analysis will be used to characterize and identify unique elements of the isolated plasmids. To determine their biological role in these bacteria, further analysis of these plasmids will examine whether they carry resistance to antibiotics or environmental toxins.

P2376**Board Number: B1058****Enzymatic regulation by filament formation: product-stimulated polymerization inhibits the enzymatic activity of CTP Synthetase.**R. Barry¹, A. Bitbol², A. Lorestani¹, E. Charles³, J.M. Hansen³, C. Habrian⁴, H. Li¹, E. Baldwin⁴, N. Wingreen⁵, J. Kollman⁶, Z. Gitai¹;¹Dept. of Molecular Biology, Princeton University, Princeton, NJ, ²Princeton University, Princeton, NJ,³Department of Anatomy and Cell Biology, McGill University, Montreal, QC, ⁴UC-Davis, Davis, CA,⁵Princeton Univ, Princeton, NJ, ⁶Univeristy of Washington, Seattle, WA

Large-scale protein localization screens revealed numerous metabolic enzymes with distinct, non-diffuse localizations. But why do these enzymes assemble into large-scale structures? Here we focus on CTP Synthetase (CtpS), which synthesizes CTP and forms filamentous structures in prokaryotes and eukaryotes. In *C. crescentus*, CtpS polymers help regulate cell shape, but *E. coli* CtpS polymers serve no obvious structural function. Here we demonstrate that CtpS uses a novel polymerization-based mechanism to harness the cooperativity of polymerization to maintain tight control of CtpS activity.

To determine why CtpS polymerizes, we developed a biochemical system to simultaneously monitor CtpS polymerization and enzymatic activity. Increased polymerization correlated with a sharp decrease in enzymatic activity, and polymer assembly was stimulated by the enzyme's product, CTP. Thus, product-induced polymerization functions to inhibit CtpS activity. To understand the mechanism underlying this assembly-mediated form of end-product inhibition, we used electron microscopy to define the structure of CtpS polymers. This structure suggests that polymerization sterically hinders a conformational change necessary for CtpS activity. Structure-guided mutagenesis and mathematical modeling further indicate that coupling activity to polymerization promotes cooperative catalytic regulation. Our hypothesis that polymerization-based regulation facilitates ultrasensitive control of nucleotide biosynthesis is supported by metabolomic analysis of CtpS polymerization disruption in vivo in *E. coli*. This type of regulation may allow cells to respond rapidly to fluctuations in nutrient availability or depletion of key intracellular molecules.

The elucidation of CtpS filament structure and its regulatory role may inform the rationale for localization of other metabolic enzymes. Additionally, the use of similar large-scale assembly-based regulation strategies by ancestral enzymes may explain the evolutionary relationship between metabolic proteins like hexokinases and structural proteins like MreB, such that CtpS could serve as a paradigm for understanding the origins of the first cytoskeletal elements.

P2377

Board Number: B1059

Role of cardiolipin in *Rhodobacter sphaeroides* cell morphology.

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Bacteria come in a variety of shapes that are formed by the orchestration of its internal cytoskeleton and external cell wall. Cell shape influences cellular functions and indicates cell health. Although it has been suggested in rod-shaped bacteria that MreB forms filamentous structures along the long axis that serve as an internal scaffold and direct the activity of penicillin-binding protein 2 (PBP2) for cell wall elongation, the morphogenetic factors that guide the formation of these macromolecular structures still remain elusive. In this study, we report that cardiolipin (CL) plays a role in the rod-shaped morphology of *Rhodobacter sphaeroides*. A CL-deficient strain of *R. sphaeroides* shows an alteration in cell shape, from rods to ellipsoids. Complementation of the CL-deficient mutant restores a rod shape. In addition, overproduction of CL in the wild-type strain of *R. sphaeroides* enhances the rod shape and leads to cell elongation. Finally, we found that in *R. sphaeroides*, rod shape formation also requires MreB and PBP2. Future work will be dedicated to understanding the interplay of these three components in *R. sphaeroides* cell elongation machinery.

P2378**Board Number: B1060****Characterization, Comparative Genomics and Genome Mining for Antibiotics and Secondary Metabolite of two Actinomycetales isolates.**R.M. Mariita¹, S. Bhatnagar², K. Hanselmann³, M. Hossain¹, M. Liles¹, A.G. Moss¹, J.L. Leadbetter⁴, D.K. Newman⁵;¹Biological Sciences, Auburn University, Auburn, AL, ²Microbiology Graduate Group, Univ California-Davis, Davis, CA, ³Plant Biology /Microbiology, University of Zurich, Zurich, Switzerland,⁴Biology/Biological Engineering, California Inst Technology, Pasadena, CA, ⁵California Inst Technol/HHMI, Pasadena, CA

Actinomycetes are ubiquitous Gram (+) bacteria commonly found to have high G+C content and best known for their metabolic by-products and novel enzymes [1]. Isolates CCMMD2014 & MRMD2014 were co-cultured from soil impacted by a rusty fire hydrant in Woods Hole, MA. The *Streptomyces* sp. and *Curtobacterium* sp. isolates were identified by marker genes for 16S rRNA, rpoB, xylose isomerase, tryptophan synthase beta chain and Cytochrome P450 monooxygenase. Both isolates showed lactic acid fermentation and urease activity. The co-isolates were separated by selective culturing with antibiotics. In addition, whole genome sequencing revealed distinct inherent metabolic pathways in each culture that allowed for mutually exclusive selective culture conditions. Assembly was done using HGAP3 with Celera8 assembler using SMRT portal [2,3]. Annotation was done using the RAST server [4], with 7540 and 3969 CDS for *Streptomyces* sp. and *Curtobacterium* sp. respectively being revealed by AMIGene and BASys [5,6]. Subsequently, antiSMASH [7], was used to predict 52 and 26 secondary metabolite biosynthetic clusters that included genes for lantipeptides, terpenes, siderophores, polyketide synthases type I and II, bacteriocin and nonribosomal peptide synthase genes for *Streptomyces* sp. and *Curtobacterium* sp. respectively. The isolates have genes of potentially beneficial traits that could help study, among others, the role of fimbrial adhesins and iron in biofilm formation and investigation on natural products.

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Cited Literature 1. Hodgson DA (2000) Primary metabolism and its control in streptomycetes: a most unusual group of bacteria. *Adv Microb Physiol* 42: 47-238. 2. Chin CS, Alexander DH, Marks P, Klammer AA, Drake J, et al. (2013) Nonhybrid, finished microbial genome assemblies from long-read SMRT sequencing data. *Nat Methods* 10: 563-569. 3. Myers EW, Sutton GG, Delcher AL, Dew IM, Fasulo DP, et al. (2000) A whole-genome assembly of *Drosophila*. *Science* 287: 2196-2204. 4. Aziz RK, Bartels D, Best AA, DeJongh M, Disz T, et al. (2008) The RAST server: Rapid annotations using subsystems technology. *BMC Genomics* 9. 5. Bocs S, Cruveiller S, Vallenet D, Nuel G, Medigue C (2003) AMIGene: Annotation of Microbial Genes. *Nucleic Acids Res* 31: 3723-3726. 6. Van Domselaar GH, Stothard P, Shrivastava S, Cruz

JA, Guo A, et al. (2005) BASys: a web server for automated bacterial genome annotation. *Nucleic Acids Res* 33: W455-459. 7. Blin K, Medema MH, Kazempour D, Fischbach MA, Breitling R, et al. (2013) antiSMASH 2.0--a versatile platform for genome mining of secondary metabolite producers. *Nucleic Acids Res* 41: W204-212.

P2379

Board Number: B1061

Fitness profiling of yeast mutants reveal unique genetic responses to spaceflight.

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Many tumors have low redox potential, with local hypoxia and hypercapnia. The decreased convection-mediated transport of nutrients, gases, and metabolic products during spaceflight may offer a microenvironment for growing cells that mimics many of the metabolic features of tumors. We proposed that molecularly barcoded yeast deletion series can provide a quantitative assessment of the effects of spaceflight on a model organism. In liquid culture yeast have no requirement for substrate adhesion or physical cell-cell interactions and their responses during spaceflight can be quantified and compared to well-established databases of ground-based stressors to allow clear examination of features that are unique to microgravity as well as features that are shared with ground-based perturbations. We screened the complete collection of 4800 non-essential yeast homozygous alleles and a collection of 5900 yeast heterozygous alleles including 1200 alleles encoding essential genes in spaceflight and ground conditions, with and without stimulation by hyperosmolar sodium chloride as a second additive stressor. The genome-wide sensitivity profiles obtained from these treatments were queried for their similarity to a compendium of drugs whose effects on yeast have been studied by chemogenomics. Spaceflight has subtle but significant effects on core cellular processes including growth control via RNA and ribosomal biogenesis, metabolism, modification and decay pathways. Significant roles for DNA repair and replication, response to pH signaling, control of gene expression and mitochondrial function were observed. Our results strongly implicate DNA and RNA damage as the major ground based analogs of spaceflight. Given the unique, and substantial radiation exposure in space, this is consistent with major radiation-mediated effects. The high concordance to the profile induced by diallyl disulfide suggests increased glutathione S-transferase, binding of electrophilic toxins, increased reactive oxygen species and change in redox state. Identification of these vital pathways can guide future experiments by suggesting environmental modifications that can bolster cellular and organismal integrity by avoiding further stress to these pathways, and secondly, by identifying drug

stresses that can exacerbate these pathway requirements in an effort to control pathological cell growth in the case of proliferative diseases. The performance of this platform is significant for spaceflight studies and promises to enable terrestrial experiments in extreme environments that will have direct application to microbial bioprocessing for manufacturing, alternative fuel development and basic research.

Muscle Structure, Function, and Disease

P2380

Board Number: B1063

Effects of Vitamin E on primary cultures of dystrophin-deficient mdx mice.

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Several studies suggest a role for free radicals in the degeneration of dystrophin-deficient muscle, as observed in Duchenne muscular dystrophy (DMD). In the present study, we investigated the effects of Vitamin E (antioxidant) on primary skeletal muscle cell cultures derived from an animal model of DMD, the mdx mice. Dystrophin-deficient muscle cells were treated with Vitamin E (7.5 μ M) for 24 hours. Untreated dystrophin-deficient muscle cells were used as control. Drug toxicity was assessed using a MTT cell viability assay. Hydrogen peroxide (H₂O₂; a marker of cellular reactive oxygen species production) was measured by using the Amplex red assay kit. 4-hydroxynonenal (4-HNE) analysis was used as a lipid peroxidation marker. The extent of the inflammatory process was assessed by TNF- α and NF- κ B levels by western blotting. Intracellular calcium concentration was indicated by the fluorescent indicator, Fluo-4. The MTT results showed that Vitamin E had no cytotoxic effect on mdx muscle cells. 4-HNE levels and intracellular calcium concentration were not altered by the Vitamin E treatment on dystrophic muscle cells. Vitamin E treatment decreased H₂O₂ production by 4% and TNF- α and NF- κ B levels by 11% and 23% respectively on mdx muscle cells. However, these reductions were not significant ($p \geq 0.05$; Anova). Based on the results, it can be hypothesized that Vitamin E was not potent enough or that the duration of treatment was too short to detect a significant positive effect on dystrophin-deficient muscle cells. Ethical approval: CEUA-IB-UNICAMP #3334-1. Support: FAPESP #2011/02474-4; #2013/17299-9.

P2381**Board Number: B1064****Effects of low-level laser therapy on inflammatory and oxidative stress parameters in dystrophic muscle fibers.**A.B. Macedo¹, D.S. Mizobuti¹, T.A. Hermes¹, L.H. Moraes¹, E. Minatel¹;¹UNICAMP, Campinas, Brazil

Duchenne muscular dystrophy (DMD) is a fatal X-linked disease that affects 1 in 3,500 child births. Reactive oxygen species (ROS) and inflammatory response play a key role in the pathogenesis of DMD. Evidence suggests that low-level laser therapy (LLLT) reduces the release of ROS, decreases the inflammatory process and increases the production of antioxidants. With this perspective in mind, the present study investigates the in vivo and in vitro effects of the LLLT on inflammatory and oxidative stress parameters in dystrophic muscle fibers of mdx, the experimental model of DMD. Primary cultures of mdx skeletal muscle cells were irradiated one time alone with laser and analyzed after 48 hours. While, in the in vivo study, the dystrophic quadriceps muscle was treated with laser three times a week for two weeks. Both studies considered C57BL/10 mice as controls. The LLLT parameter used was 830 nm wavelengths at 5 J/cm² fluence. In the in vitro study, cell viability was assessed by MTT assay; hydrogen peroxide (H₂O₂; a marker of cellular ROS production) was measured by using the Amplex red assay kit and intracellular calcium concentration was indicated by the fluorescent indicator, Fluo-4. In the in vivo study, dihydroethidium (DHE) staining for ROS detection was used. In the in vivo and in vitro studies, the extent of the inflammatory process was assessed by TNF- α level. The laser treatment in dystrophic muscle cells improved the cell viability (15%), significantly decreased H₂O₂ production (25%; $p \leq 0.05$, Anova) and reduced the TNF- α level (by 19%; $p \leq 0.05$, Anova). In the dystrophic quadriceps muscle, LLLT significantly reduced the TNF- α level (8%; $p \leq 0.05$, Anova) and DHE staining (by 50%; $p \leq 0.05$, Anova). These results suggest that the laser decreased inflammatory process and oxidative stress in dystrophic muscle fibers, indicating that LLLT could be a helpful alternative therapy to be associated with other pharmacological treatments. Ethical approval: CEUA-IB-UNICAMP #2974-1. Support: CAPES; FAPESP #2011/02474-4.

P2382**Board Number: B1065****Gamma-sarcoglycan is required for the response of archvillin to mechanical stimulation in skeletal muscle.**J. Spinazzola¹, T.C. Smith², M. Liu³, E.J. Luna², E.R. Barton¹;¹Anatomy Cell Biology, University of Pennsylvania, Philadelphia, PA, ²Cell Developmental Biology, University of Massachusetts Medical School, Worcester, MA, ³Physiology, University of Pennsylvania, Philadelphia, PA

In skeletal muscle, mechanotransduction is mediated in part by the dystrophin-glycoprotein complex (DGC), an assembly of proteins in the sarcolemma that links the cytoskeleton and the extracellular matrix. Mutations of DGC components cause several forms of muscular dystrophies, progressive neuromuscular disorders characterized by severe muscle weakness. Gamma-sarcoglycan (γ -SG) deficiency causes Limb Girdle muscular dystrophy type 2C and is also a secondary consequence of dystrophin mutation in Duchenne muscular dystrophy. While dystrophin deficiency causes contraction-induced mechanical damage that leads to pathology, deficiency in γ -SG alone is sufficient to induce skeletal muscle degeneration and signaling defects, disrupting muscle adaptation to mechanical load. However, mechanisms of γ -SG mediated signaling and the proteins involved are poorly understood.

We investigated γ -SG as a component of the load-sensing machinery of muscle cells, and through yeast two-hybrid screens, identified the muscle-specific protein archvillin (AV) as a γ -SG and dystrophin interacting protein. Archvillin is an attractive candidate because it colocalizes with dystrophin at the sarcolemma, and its isoform, smooth muscle AV, associates with ERK in a stimulus-dependent manner. Archvillin expression and localization were analyzed by IB, qRT-PCR and IHC in hindlimb muscles of wild-type (C57), γ -SG -null (*gsg*^{-/-}), and dystrophin deficient *mdx* mice. Archvillin protein and mRNA expression were significantly upregulated in *gsg*^{-/-} muscle, but were similar to C57 levels in *mdx* muscle. In muscle cross-sections, AV was upregulated at the sarcolemma in *gsg*^{-/-} muscle, but absent in *mdx* muscle. Reintroduction of γ -SG in *gsg*^{-/-} muscle by rAAV injection normalized archvillin levels toward that of C57. Mechanical perturbation of TA muscles with an in situ eccentric contraction protocol revealed that AV associates with P-ERK in a stimulus-dependent manner, but this interaction was lost in *gsg*^{-/-} and *mdx* muscle. Nuclear/cytoplasmic fractionation studies showed that nuclear localization of P-ERK1/2 increased in *gsg*^{-/-} and *mdx* muscle after eccentric contraction.

In this study, we established that AV is a new player in mechanical signal transduction in muscle, and that its localization and actions require the presence of dystrophin and the sarcoglycans, respectively. Mechanical fragility may not be the only deficit contributing to the dystrophic process, and AV may contribute to the aberrant signaling observed in dystrophic muscle. Analyzing potential components of γ -SG-mediated mechanotransduction will be an important step in understanding the pathways leading to dystrophic pathology, and may contribute to the development of therapies for muscular dystrophies.

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P2383**Board Number: B1066****Replacement of myosin molecules in thick filament of cultured skeletal muscle.**E. Ichimura¹, Y. Yasukawa¹, K. Ojima², J. Wakamatsu¹, T. Nishimura¹;¹Hokkaido University, Sapporo, Japan, ²Animal Products Res. Div., NARO, ILGS, Tsukuba, Japan

Myosin is one of the major myofibrillar proteins of skeletal muscle. More than 300 myosin molecules assemble into a bipolar thick filament in the sarcomere. We have previously shown that myosin molecules in thick filaments are vigorously exchangeable in cultured myotubes using fluorescence recovery after photobleaching (FRAP) technique. However, it is still unclear how myosin molecules in thick filaments are replaced. In this study, we investigated the replacement of myosin molecules in myotubes, when protein biosynthesis was inhibited. Exogenously expressed eGFP-tagged myosin heavy chain 3 (eGFP-Myh3) were precisely incorporated into the A-band of the sarcomeres. We carried out FRAP experiments in the presence of cycloheximide (CX) to inhibit protein biosynthesis. The fluorescence recovery of eGFP-Myh3 in myotubes treated with CX for an hour was not different from that of control without CX-treatment. However, the fluorescence of eGFP-Myh3 in myotubes treated with CX for ten hours recovered to a less extent compared with the control group. This result suggests that myosin molecules in thick filaments were exchanged largely by myosin molecules which might exist in the cytosol adjacent to myofibrils. To address this, we examined myosin molecules replacement in myotubes treated with streptolysin O (SLO) which makes pores in the cell membrane. The fluorescence recovery of eGFP-Myh3 in myotubes treated with SLO was suppressed compared to the control without SLO-treatment. This might be caused by the decrease in the amount of myosin molecules in the cytosol due to the outflow of myosin molecules that were not incorporated into thick filaments from myotubes. Our data suggest that myosin molecules in the thick filament are actively replaced with myosin molecules that exist in the cytosol of skeletal muscle cells.

P2384**Board Number: B1067****Effects of glucose concentrations on myoblast differentiation, muscle fiber composition and CB1 receptor expression.**D. Zhao¹, A.R. Conley²;¹Lycoming College, Williamsport, PA, ²Biology, Lycoming College, Williamsport, PA

Glucose is most important simple sugar for the human body, and changes in extracellular and intracellular glucose concentrations can have a dramatic effect on a cell's morphology and function. Studying these effects is of high importance due to the increasing presence of type II diabetes in the United States' population. In this study, C2C12 cells, muscle myoblasts, were differentiated at various concentrations of glucose (10mM, 15 mM, and 25mM) in order to determine how glucose levels affected differentiating muscle stem cells. RNA was isolated after one day of differentiation and after

the cells were fully differentiated at day six. RT-qPCR was run to determine how differentiation time and glucose concentration affected gene expression levels. Muscle fiber composition was studied using myosin heavy chain (MHC) primers and the three primers were used to determine the gene expression levels of the cannabinoid 1 receptor (CB1R). Gene expression levels of CB1R and MHCIIx (fast muscle fiber) increased dramatically in the high glucose sample of fully differentiated cells. It can be suggested that altering glucose levels is enough to alter the muscle fiber composition of skeletal muscle cells. Further studies need to be performed to determine if there is a relationship between the increase in CB1R expression and the increase in fast muscle fibers (MHCIIx expression).

P2385

Board Number: B1068

IGF-1 gene induced by ingestion of soy peptides inhibits skeletal muscle atrophy.

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A result of examining the effects of soy peptides intake to muscle atrophy caused by no-load model, it was found that the soy peptides, there is a growth-promoting action of skeletal muscle atrophy and inhibitory effect on skeletal muscle. In this experiment, male ICR mice were used (body weight 42.3 ± 2.7 g average, Japan SLC, Inc.) and 20 animals. In an environment under 24 ± 1 °C room temperature, a 12-hour day-night reversal light-dark cycle, and ad libitum drinking water and food and were housed in individual cages for 7 days. Diet using the MF (Oriental Yeast Co., Ltd.) was used (Fuji Oil Co., Ltd.) soy peptides AM which was prepared in 175 mg / mL and pure drinking water. The molecular weight of the soybean peptide to be used in this experiment was 5000 or less. Pure water intake group (the "Group W"), pure water intake + tail-suspension group (hereinafter referred to as "WTS group"), soy peptides Newt AM high intake group (hereinafter "AM group") soy peptides High Newt AM ingested mouse + I was divided into four groups of five mice tail suspension group ("AMTS group" below). I was using the tail suspension method Emily Morey was devised as a method of inactivity. Seven days after tail suspension, were sacrificed under anesthesia each mouse group W, WTS group, AM group, the AMTS group, were collected each plantaris muscle is a fast muscle and soleus muscle is a slow muscle and weight, electronic balance I was measured by METTLER TOLEDO Co., Ltd.). Myofibrillar protein content was measured according to the Bradford method. After staining with HE Frozen muscle sections were prepared on a cryostat, the area of the cells was measured under a microscope. Furthermore, in order to elucidate the mechanism of the suppression muscle atrophy, a study was conducted by focusing on the behavior of IGF-1. The growth during development of skeletal muscle and skeletal muscle hypertrophy time, IGF-1 is significantly increased, are generally known. The results of these experiments, an increase in IGF-1 concentration was found by AM administration clearly. That the gene expression of the IGF-1 increases by more AM uptake revealed. The increase in IGF-1R was also observed. It is inferred from the above results, a gene of IGF-1 is expressed by AM administration, and IGF-1 is synthesized by the autocrine mechanism, and is suppressed muscle atrophy.

P2386**Board Number: B1069****PEDF and PEDF-derived peptide promotes skeletal muscle regeneration by its mitogenic effect on muscle progenitor cells.**Y. Tsao¹;¹Department of Ophthalmology, Mackay Memorial Hospital, Taipei, Taiwan

Muscle injury triggers an intrinsic regenerative process that includes the proliferation of satellite cells which gives rise to myoblasts before the eventual differentiation into myotubes. In cultured C2C12 myoblast cells, pigment epithelial-derived factor (PEDF) and synthetic PEDF short peptide (PSP; residues Ser93-Leu112) stimulated mitogenesis together with the raise of cyclin D1 expression. PEDF evoked phosphorylation of ERK1/2, Akt and STAT3. Blockade of activity of ERK, Akt or STAT3 by pharmacological inhibitors attenuated PEDF effects on the induction of cell proliferation and cyclin D1 expression. Muscle regeneration promoted by PSP was confirmed by increased myotube formation in a rat model of myonecrosis induced by bupivacaine. BrdU pulse-labeling assay demonstrated that PSP stimulated primary rat satellite cell proliferation in injured soleus muscle and in cell culture and the process is mediated through ERK, Akt and STAT3 signaling. Our results extend the understanding of the biological activity of PEDF on modulating muscle progenitors. Our data suggest that the PEDF peptide benefits muscle repair by mechanism involving its mitogenic effect on muscle progenitors.

P2387**Board Number: B1070****Transforming growth factor type beta 1 (TGF- β 1) induces skeletal muscle atrophy by a mechanism dependent of NADPH oxidase-induced reactive oxygen species.**J. Ábrigo¹, M.G. Morales¹, F. Simon¹, C. Cabello-Verrugio¹;¹Departamento de Ciencias Biológicas, Universidad Andres Bello, Santiago, Chile

TGF- β 1 signaling pathway play an important role in skeletal muscle homeostasis and its expression is linked to normal processes in skeletal muscle such as growth, differentiation, regeneration, and stress response. Recently, TGF- β 1 is linked to skeletal muscle atrophy. Skeletal muscle wasting is characterized by the loss of muscle mass, decline in strength and the increase of myofibrillar proteins degradation, mainly through the ubiquitin-proteasome system (UPS). Two specific muscle E3 ubiquitin-ligases, atrogin-1 and MuRF-1, are upregulated in skeletal muscle atrophy, whose target of degradation are myofibrillar proteins, such as myosin heavy chain (MHC). Recent data shown that TGF- β 1 increase the atrogin-1 expression. However the mechanism involved in TGF- β 1-induced atrophy has not been elucidated. One of the mechanisms induced by TGF- β 1 that could participate in muscle atrophy is the increase of reactive oxygen species oxidative (ROS) levels mediated by NADPH oxidase (NOX). Based on this we decided to evaluate the participation of ROS dependent on NOX in the generation of skeletal

muscle atrophy induced by TGF- β 1. C2C12 myotubes were treated with TGF- β 1. The protein levels of atrogen-1, MuRF-1 and MHC was evaluated by western blot. The diameter of myotubes was determined by indirect immunofluorescence for MHC. The production of ROS induced by TGF- β 1 was evaluated by the fluorescent probe DCF. To evaluate the ROS participation in the atrophy generation induced by TGF- β 1, myotubes was incubated in absence or presence of anti-oxidant N-acetylcystein (NAC), while that apocynin (NOX inhibitor) was used for determinate the participation of NOX. Our results shown that TGF- β 1 regulates the levels of atrogen-1 and MuRF-1 during the differentiation, in a differential fashion, showing an increment of both E3 ubiquitin-ligases at early stages of differentiation (day 2), while MuRF-1 levels are only increased in late stages (day 5). At day 5, TGF- β 1 produce a decrease in the levels of MHC and the myotubes diameter and induce an increase of the ROS levels, mediated by NOX. Finally, we determined that the diminution of ROS prevent the atrophic effect induced by TGF- β 1 in myotubes evaluated by MuRF-1 and MHC levels. The results obtained, provide new evidence about the atrophic effect of TGF- β 1 in skeletal muscle cells, and demonstrated that NOX-induced ROS is a key regulator of the TGF- β 1-dependent atrophy in skeletal muscle cells and represent a new therapeutic target against skeletal muscle atrophy.

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P2388

Board Number: B1071

The regulation of muscle structure and function by Mio/dChREBP in *Drosophila*.

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All cells require energy to perform their specialized functions. Muscle is particularly sensitive to the availability of nutrients due to the high-energy requirement for muscle contraction. Therefore the ability of muscle cells to obtain, store and utilize energy is essential for the function of these cells. Mio, the *Drosophila* homolog of carbohydrate response element binding protein (ChREBP), has recently been identified as a nutrient responsive transcription factor important for triglyceride storage in the fly fat body. However, the function of *Mio* in muscle is unknown. In this study, we characterized the role of Mio in controlling muscle function in adult flies. Decreasing *Mio* levels using RNAi specifically in muscle leads to a flight defect and altered myofibril structure in the indirect flight muscles as shown by electron microscopy. *Mio* RNAi flies also have increased glycogen storage in their thoraxes as well as altered glycolytic and glycogenic gene expression, potentially contributing to the impairment in flight muscle function observed in these flies. Together, these data indicate a novel role for Mio to control muscle structure and metabolism and may provide a molecular link between nutrient availability and muscle function.

P2389**Board Number: B1072****Dynamics of secreted proteins from skeletal muscle cells during muscle differentiation.**K. Ojima¹, M. Oe¹, I. Nakajima¹, S. Muroya¹, T. Nishimura²;¹Animal Products Res. Div., NARO, ILGS, Tsukuba, Japan, ²Hokkaido University, Sapporo, Japan

Skeletal muscle cells drastically change their phenotype after differentiation, *i.e.*, proliferating mononucleated cells shift to multinucleated myotubes or myofibers. Growth factors are critical in this muscle differentiation process. Recently, it has been shown that skeletal muscles secrete growth factors, called myokines. However, it is not clear what type of protein is released from skeletal muscle cells during differentiation. To address this, we evaluated the dynamics of secreted proteins using mass spectrometry combined with an iTRAQ[®] labeling method to identify proteins quantitatively and comprehensively. Secreted levels of proteins were compared at four different culture time points, including one proliferating and three differentiating stages. We detected more than 400 proteins in cultured medium from muscle cells and approximately 8% of them were categorized as secreted proteins based on a gene ontology classification. The levels of secreted proteins associated with promoting myotube formation or enhancing vascularization peaked during early muscle differentiation, whereas proteins contributing to repellent activity against nerve cells or suppression of adipogenic differentiation decreased in quantity after muscle differentiation. Our data suggest that muscle cells secrete different types of proteins at different myogenic developmental stages as a tool to communicate with other types of cells that organize skeletal muscle tissues *in vivo*.

P2390**Board Number: B1073****Lipopolysaccharide-induced skeletal muscle atrophy is prevented by Angiotensin (1-7) via Mas receptor.**M.G. Morales¹, H.H. Olguin², E. Brandan², F. Simon¹, C. Cabello-Verrugio¹;¹Departamento de Ciencias Biologicas, Universidad Andres Bello, Santiago, Chile, ²Departamento de Biologia Celular y Molecular, Pontificia Universidad Catolica de Chile, Santiago, Chile

During skeletal muscle atrophy there is a severe loss of muscle mass, a decrease on muscle strength and myosin heavy chain (MHC) levels, an up regulation of muscle-specific ubiquitin 3 ligases atrogin-1 and MuRF-1. Sepsis is a cause of muscle wasting. In a mice model of lipopolysaccharide (LPS)-induced sepsis, skeletal muscle atrophy is observed and characterized by the activation of p38 MAPK pathway. Angiotensin (1-7) [Ang (1-7)], the main peptide of non-classical renin-angiotensin system, exerts its actions through the Mas receptor. We recently demonstrated that: a) Ang (1-7) has anti-atrophic activity in a model of muscle wasting induced by angiotensin II (Ang-II) through a mechanism dependent on AKT;

b) Ang (1-7) decreases the p38 MAPK activation induced by Ang-II in skeletal muscle cells. Therefore, we want to evaluate the effect of Ang (1-7) and Mas receptor on the LPS-induced skeletal muscle atrophy, a model of muscle wasting independent of Ang-II.

For this, C2C12 myotubes and isolated skeletal muscle fibers were incubated with LPS in absence or presence of Ang (1-7) and Mas receptor antagonist (A779). C57BL/10J mice were injected intraperitoneally with LPS and infused through osmotic pumps with Ang (1-7) and A779, and gastrocnemius and tibialis anterior muscles were analyzed. To evaluate muscle atrophy in vivo, we determine the fiber size by H&E staining and the muscle strength by electrophysiological measurements in isolated muscles. For analysis in vitro the myofiber and myotube diameter was determined. In addition, in vitro and in vivo analysis of atrogen-1 and MuRF-1 gene expression was performed by RT-qPCR, and MHC and p38 MAPK (phosphorylated and total) levels were evaluated by western blot. We determined that Ang (1-7), through the Mas receptor, decreases LPS-induced skeletal muscle atrophy, preventing the diminution in fiber diameter and maximal isometric force of the gastrocnemius and tibialis anterior muscle, resulting in a 35% increase in muscle strength compared to mice treated with LPS. Concomitantly, Ang (1-7) inhibits the decreased in MHC levels and abolished the induction of the atrogen-1 and MuRF-1 gene expression. Ang (1-7) decreased the phosphorylation of p38 MAPK induced by LPS in vivo and in vitro, suggesting that the possible mechanism through Ang (1-7) exerts its anti-atrophic effect would involve, at least, the participation of p38 MAPK. The results suggest that Ang (1-7) is a molecule with anti-atrophic properties in a model induced by LPS that is independent on Ang-II, and could be further considered as a new possible therapy to improve muscle mass in a septic condition. Funding: Association-Francaise Contre Les Myopathies AFM #16670; FONDECYT #1120380, 3130593, 1121078; IMII #P09-016-F and UNAB DI-281-13/R.

P2391

Board Number: B1074

Hyperlipidemia inhibits Sca1/VEGFR2 progenitor cell mobilization and angiogenesis in ischemic skeletal muscle in ApoE^{-/-} mice and suppresses SDF/CXCR4 signaling pathway.

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Rationale: Peripheral artery disease (PAD) has multiple risk factors such as hyperlipidemia. Hyperlipidemia impaired angiogenesis after ischemic injury leading to decreased blood flow recovery. Vascular progenitor cells (VPCs) are currently considered as an important participant in the process of angiogenesis. Objective: To examine the effect of hyperlipidemia on VPCs, elucidate the underlying mechanism in which impaired VPC biological function result in exasperated new vessel formation after ischemia. Methods and Results: Male ApoE^{-/-} mice were fed with standard diet (ApoE group) or 2% high-cholesterol diet (ApoE/HF group) at the age of 8 weeks for 12 weeks. Wild type mice were used as control group (WT group). Hind limb ischemia (HLI) was surgically induced in all animals. Dietary ApoE/HF group revealed elevated total and LDL cholesterol levels and remarkable aortic lesion

compared with WT and ApoE group. Laser Doppler blood flow analysis showed significant decrease in the ischemic/normal limb blood flow ratio in the ApoE/HF group compared with WT and ApoE group (P

P2392

Board Number: B1075

Propolis attenuates the capillary regression and anti-angiogenic factor expression in unloading-induced atrophy of skeletal muscle.

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Aim: The capillary regression in skeletal muscle with a chronic decrease in activity such as unloading is related to a dysfunction of endothelial cells induced by a decrease of oxygen demand and increase of oxidative stress. Propolis is natural polyphenol-rich substance collected by honeybees that may be expected to attenuate capillary regression in atrophied skeletal muscle. The purpose of the present study was to investigate the preventive effects of propolis administration on capillary regression in the skeletal muscle during hindlimb unloading. **Methods:** Twenty-eight adult male Wistar rats were assigned randomly either to a control, control plus propolis administration, hindlimb unloaded or hindlimb unloaded plus propolis administration group for 14 days. The three-dimensional capillary network of soleus muscle was visualized using a confocal laser microscopy, and the capillary volume was measured. In addition, the pro- and anti-angiogenic factors, and oxidative stress maker were determined. **Results:** The capillary volume in atrophied muscle was lower than that in control. Propolis administration attenuated the decrease of capillary volume. In addition, the expression level of thrombospondin-1, as an anti-angiogenic factor, and SOD-1 in propolis administrated muscle were attenuated by propolis administration. **Conclusion:** These data suggest that propolis may be an effective treatment to counter the detrimental effects of a chronic decrease in skeletal muscle use on the capillary. **Keywords** anti-angiogenic factor, antioxidant, capillary, muscle atrophy, oxidative stress.

P2393

Board Number: B1076

Osteoactivin, Inducible Heat Shock Protein 70 and Transforming Growth Factor β 1 Expression in Skeletal Muscle of High Repetitive High Force Reaching Rats in Association with Anti-Inflammatory Drugs.

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Osteoactivin (OA) was proposed as a potential repair protein involved in tissue turnover during regeneration and degeneration. Previous studies have shown OA as a negative regulator of

inflammation that attenuates macrophage inflammatory responses by decreasing production of pro-inflammatory cytokines. Although, OA is thought to be involved in tissue injury and repair, its response to overuse has yet to be studied. We have previously developed a rat model of repetitive reaching of overuse/overload that induces injury, inflammatory and fibrotic changes in musculoskeletal tissues. We anticipate that OA will increase in the overloaded tissues. We hypothesize that OA is functioning as a repair molecule in this model and increases in musculoskeletal tissues as microtrauma increases, in order to stimulate tissue turnover and repair. We used our model of overuse/overload in association with anti-inflammatory drugs to evaluate OA's link to inflammation with prolonged overuse. In addition, we examined other proteins involved in tissue repair such as hsp72 and TGF β 1. Young adult female Sprague-Dawley rats trained for approximately 6 weeks (10 min/day, 5 days/week; TR-HF) and then performed a high repetition high force (HRHF) task with 60% maximum pulling force for 6, 12, and 18 weeks (2 hours/day, 3 days/week). Results were compared to age-matched normal control (NC) or food restricted (FRC) rats. At the end of HRHF task week 4, cohorts of HRHF rats were administered either ibuprofen in drinking water daily for 2 weeks, or were treated with an anti-rat TNF- α drug (IP injections) across 2 weeks. Western blot analysis of flexor digitorum muscles of untreated 6-week HRHF rats showed higher expression levels of OA than TR-HF and control rats. ELISA showed a significant increase in hsp72 and TNF- α in muscles of 6-week HRHF rats without anti-inflammatory treatment, compared to TR-HF and control rats. In contrast, 6-week HRHF rats treated with the anti-TNF- α drug in their final 2 weeks showed reduced OA levels to baseline NC levels. Treatment of 6-week HRHF rats with ibuprofen for 2 weeks lead to higher OA levels. However, both anti-TNF- α and ibuprofen treatments decreased the 6-week HRHF muscle hsp72 levels to baseline TR-HF levels and reduced histological signs of muscle fibrosis. Prolonged performance of the HRHF task to 18 weeks without anti-inflammatory treatment showed increased OA and hsp72 in muscles concomitant with increased TGF β 1 and muscle fibrosis. The 18-week HRHF muscles also showed decreased TNF- α expression, indicating a resolution of muscle inflammation by 18 weeks. Thus, performance of highly repetitive tasks was associated with an increase in several anabolic proteins in flexor digitorum musculotendinous tissues, some associated with fibrotic repair, particularly when combined with high force. The reduction of OA after anti-TNF- α treatment indicates that TNF- α , but not cyclooxygenases, plays a role in OA production. The continued increase of OA after the task-induced increase in TNF- α (seen at 6 weeks) had resolved (18 weeks), suggests that other factors also induce OA production.

P2394

Board Number: B1077

Changes in Golgi elements are responsible for the disordered microtubule network of DMD muscle fibers.

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Microtubules have been implicated in the pathology of Duchenne muscular dystrophy (DMD), a severe muscle disease that results from the absence of the protein dystrophin. This protein links the muscle

plasmalemma to cytoskeletal proteins such as actin, intermediate filaments, and tubulin/microtubules. Microtubules of mouse fast muscles form a remarkable orthogonal network near the muscle plasmalemma but in the dystrophin-less mdx mouse, a popular mouse model for DMD, the organization of microtubules is very much disturbed, in agreement with the notion that dystrophin guides microtubules. We have recently demonstrated that the microtubule network in wild-type (WT) mice is formed from dynamic microtubules nucleated from Golgi elements and forming bipolar longitudinal and transverse bundles (Oddoux et al., J.Cell Biol. 2013, 203, 205). We are now investigating dynamics and nucleation of mdx microtubules. We find only small differences in the growth rates measured by tracking EB3-GFP and GFP-tubulin in live mdx fibers, and find no differences in classic parameters of microtubules such as pause frequencies and duration, shrinking rates, and distance covered by growing tips. In contrast, we observe differences in the directionality of microtubule growth (the angle of microtubule growth in relation to the fiber axes), quantitated by a microtubule-targeted software package (Liu & Ralston, 2014, Cytoskeleton, 71, 230). Furthermore, these differences seem to exist practically from the moment growing microtubules emerge from the static Golgi elements, as shown in experiments following microtubule recovery from nocodazole. We have also proceeded to examine the organization of the myofibrillar core of the muscle fibers which also contains microtubules and Golgi elements and may contribute to DMD pathology, especially in fibers with rows of central nuclei characteristic of muscle regeneration. We had so far focused our work mostly on the cortical microtubule layer, in direct contact with dystrophin. Whereas WT fibers show a three-dimensional network of transverse and longitudinal microtubules, mdx fibers show a decrease in transverse and an increase in longitudinal microtubules. In summary, this new work suggests that positioning and orientation of the Golgi elements (first shown to be abnormal in mdx by Percival et al., 2007, Traffic 8,1424), play an essential role in the organization of muscle microtubules and indicate that the absence of dystrophin at the muscle plasmalemma translates into differences in cytoskeletal organization that affect the core as well as the surface of the fibers.

P2395

Board Number: B1078

Capillary and metabolic actions of exercise in skeletal muscle of type 2 diabetes.

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Purpose: Diabetes affects the peripheral vascular bed. Exercise may play a role of countermeasure in diabetic mitochondrial dysfunction and microangiopathy. Therefore, the purpose of the present experiments was to study the effects of exercise on capillary volume, metabolic activity and expression of the angiogenic growth factors and angiopoietins in the skeletal muscle of diabetes. Methods: Male Goto-Kakizaki (GK) spontaneously diabetic rats and age-matched wild-type Wistar rats were divided into three groups: diabetes (GK), diabetes plus exercise (GK +Ex), and sedentary control. The exercise consisted of endurance training at low-aerobic intensity (plasma lactate <~2mmol/l) and included five 60

min sessions per week for 3 weeks. The three-dimensional capillary network of soleus muscle was visualized using a confocal laser microscopy, and the capillary volume was measured. metabolic activities were determined by succinate dehydrogenase (SDH) and PCG-1 α expression. In addition, the mRNA levels of angiogenic factors were determined by TaqMan probe-based real-time PCR. Results: The level of plasma glucose was higher in diabetic GK rat as compared to control. There was no significant difference in capillary-to-fiber ratio among GK, GK+Ex and control muscles. Although the capillary volume in diabetic muscle was lower than that in control, the exercised muscle capillary was attenuated toward to the level of control. Thus, exercise had effect on capillary volume in diabetic muscle. SDH activity, PCG-1 α expression in exercised muscle were higher than those in sedentary diabetic muscle. The expression levels of angiogenic factors were lower in GK than in control muscle. However, those were attenuated toward to the levels of those in GK+Ex muscle. Conclusion: These results suggest that exercise reduces the microcirculatory complication and improves the mitochondrial activity in skeletal muscle associated with type 2 diabetes.

P2396

Board Number: B1079

Investigation of the wound healing process of adult *Danio rerio* wildtype zebrafish.

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Danio rerio (zebrafish) share many physiological and genetic characteristics with humans, making them an attractive model system for scientific research. Zebrafish have been shown to completely regenerate significant portions of heart, fin, and tail tissues without loss of function or formation of permanent scar tissue. However, zebrafish have not yet been established as a model to study regeneration in skeletal muscle and surrounding tissues. Specifically, the healing response following deep tissue burn puncture wounds has not yet been described. We have hypothesized that zebrafish should completely regenerate skeletal muscle and surrounding tissues in response to this type of injury—making them an interesting wound-healing model. Our investigations have initially focused on determining the length of time necessary for zebrafish to fully recover from deep tissue burn puncture wounds. We first standardized methods for introducing a hot puncture wound completely through the myotomal muscle of the zebrafish, one millimeter below the dorsal fin. Healing was tracked by photography of wounded fish daily until healing appeared complete upon gross examination. Our results indicate that deep tissue burn puncture wounds of this type require approximately thirty days to heal, with minimal to no external scarring visible. We have also screened wounds for infection that occurs during the healing process, and have begun to identify the endogenous bacteria that cause these infections. Follow up studies include time point sampling for analysis of the wound healing process at tissue and molecular levels, as well as investigation of biofilm formation and specific identification of bacterial species causing infection during the healing process. Our preliminary results support the use of zebrafish as a model to

investigate cellular and molecular regeneration and healing processes following deep tissue burn puncture wounds. Findings could translate into applications for treatment of burn puncture wounds to skeletal muscle in humans, such as those inflicted during military combat.

P2397

Board Number: B1080

Biochemical analysis of SERCA1b, SERCA1 splice variants related in Myotonic Dystrophy Type 1.

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SERCA(sarco/endoplasmic reticulum Ca²⁺-ATPase) is a representative member of P-type ATPase and transports Ca²⁺ from cytosol to lumen. It is crucial for maintaining the intracellular Ca²⁺ homeostasis. SERCA is generally expressed in various types of cell, and its mutations lead to Brody disease and Darier disease as reported. SERCA1 is specifically expressed in fast twitch skeletal muscle. SERCA1 has two isoforms produced by alternative splicing of exon 22, which is included in SERCA1a but not in SERCA1b. Since exon 22 contains a stop codon, SERCA1a is the shorter isoform of 994 amino acids, and SERCA1b consists of 1001 amino acids with the last eight amino acids different. SERCA1b has not been well characterized in contrast to well studied SERCA1a. SERCA1b is expressed only in neonatal stage, suggesting the importance of splicing regulation of SERCA1. In addition, in Myotonic Dystrophy Type 1 (DM1), a genetic disease, multiple genes are aberrantly spliced including that of SERCA1. Furthermore, disturbance of intracellular Ca²⁺ homeostasis has been reported in DM1. Thus, we hypothesized that aberrantly expression of SERCA1b in DM1 patients is an origin of abnormal intracellular Ca²⁺ concentration. To examine this possibility, we first conducted a western blot analysis on skeletal muscle tissue of DM1 model mice, and found that SERCA1b, normally expressed only in new born mice, is aberrantly expressed in adult mice. Next, we tried to dissect functional differences between the two isoforms by overexpressing SERCA1a and SERCA1b in HEK293 cells, and found that both ATPase and Ca²⁺ transport activities of SERCA1b are approximately half those of SERCA1a. The apparent affinity for ATP and that for Ca²⁺ are approximately the same. Thus, aberrantly expression of SERCA1b may be an origin of the intracellular high Ca²⁺ concentration in DM1.

P2398**Board Number: B1081****G-CSF attenuated heart damage in mice exposed to repetitive β -adrenergic stimulation, potentially through the participation of bone marrow mobilized stem cells.**B. NIETO-LIMA^{1,2}, V. GUARNER-LANS², F.A. MASSO-ROJAS², A. PAEZ-ARENAS², G. ZARCO-OLVERA³, A. CANO-MARTINEZ²;¹Posgrado en Ciencias Biologicas, Universidad Nacional Autonoma de Mexico, MEXICO CITY, Mexico,²Department of Physiology, Instituto Nacional de Cardiologia - Ignacio Chavez, MEXICO CITY, Mexico,³Department of Pharmacology, Instituto Nacional de Cardiologia - Ignacio Chavez, MEXICO CITY, Mexico

Fibrosis, a major feature of remodeling in damaged hearts, leads to heart failure and death of organisms. On the other hand, heart damage results in the secretion of cytokines, e.g. granulocyte colony-stimulating factor (G-CSF), capable of mobilizing neutrophils and stem cells from bone marrow. Still, this mobilization is not enough to repair the heart. Exogenous G-CSF improves heart function in animals with acutely induced heart damage; however heart failure results of multiple events of heart damage. We evaluated the effect of G-CSF on the heart of mice exposed to repeated β -adrenergic stimulation (isoproterenol, ISO) to induce heart damage. Four groups were formed and treated with saline solution (control), ISO (5 μ g/kg/d, 7d, s.c), G-CSF (300 μ g/kg/d, 4d, s.c), or ISO (3d)+ISO/G-CSF (4d). Half of the mice were sacrificed 24h after treatments and the others 7d after. Fibrosis was quantified in longitudinal heart criosections using Masson's trichrome stain. Ventricular hypertrophy (VH) was measured to confirm heart damage. Number of stem cells from bone marrow (BM) and peripheral blood (PB) was estimated with the colony forming unit (CFU) assay. Neutrophils were quantified in peripheral blood smears. Isoproterenol (24h) induced the highest percentage of fibrosis, VH and colonies from BM; while neutrophils increased 2.4-fold. At 7d, VH persisted, while fibrosis diminished 8.22% and neutrophils decreased 31.9% compared to ISO (24h). Treatment with ISO+G-CSF lowered fibrosis at 24h (12.3%) and 7d (26.0%) compared to ISO (24h). VH had the same tendency, but it was not different. Colonies from PB were the highest among all groups. Neutrophils increased 3.2-fold (24h) and decreased 26.5% at 7d. An inverse relationship between colonies from BM and PB were found with both treatments at 24h (ISO and ISO+G-CSF). Endogenous β -adrenergic stimulation releases catecholamine, improving peripheral tissue perfusion. However overproduction causes heart damage. Our results show that G-CSF (300 μ g/kg/d) reduces fibrosis, but not VH, suggesting that this cytokine partially attenuated cardiac remodeling induced with repetitive β -adrenergic stimulation. Moreover, fibrosis reduction could be time-dependent. This antiremodeling effect may be related to bone marrow mobilized stem cells; since heart damage significantly increased only colonies derived from BM, meanwhile, with G-CSF the increment was more evident in those from PB. CFU assay and neutrophils also suggest that heart damage promotes mainly the proliferation of stem cell in BM and the release of neutrophils, while the cytokine, at this dose, could be mobilizing stem cells to the circulation. However, further studies are still necessary for a better understanding of the biology of heart damage, G-CSF and stem cells.

Adipocytes and Metabolism

P2399

Board Number: B1082

Diacylglycerol kinase ϵ -KO mice are susceptible to high fat diet-induced obesity and adipose tissue-specific insulin resistance.

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Insulin-responsive organs, including adipose tissue, liver and skeletal muscle, play a central role in the metabolism of glucose and lipid. Understanding of its detailed mechanism is important since we know that high fat diet is linked to metabolic diseases such as obesity, fatty liver and type II diabetes. Diacylglycerol (DG) is an intermediate product of various lipids and represents a precursor of triglyceride (TG) synthesis. It also serves as a signaling molecule that activates DG-responsive proteins such as protein kinase C (PKC). In fact, novel PKC (nPKC) is shown to be involved in tissue-specific insulin resistance. PKC ϵ and PKC θ hinder appropriate insulin signaling in liver and fat/skeletal muscle, respectively. Therefore DG-PKC signaling may play a pivotal role in the pathogenesis of metabolic diseases. Diacylglycerol kinase (DGK) participates in lipid metabolism through phosphorylation of DG to phosphatidic acid. In this study, we fed DGK ϵ -knockout (KO), DGK α -KO and wild type (WT) mice with high fat diet (HFD) for 40 days and examined glucose and lipid metabolism. HFD-fed DGK ϵ -KO mice showed increased body weight gain and peri-epididymal white adipose tissue (WAT) mass compared with DGK α -KO and WT mice. Glucose tolerance test demonstrated that DGK ϵ -KO mice are defective in glucose metabolism under HFD-fed conditions. Histological examination of WAT revealed an increased size of adipocytes in DGK ϵ -KO mice compared with that in WT mice. In immunoblot analysis, expression of adipose triglyceride lipase, an enzyme catalyzing lipolysis of TG into DG, was reduced in WAT of HFD-fed DGK ϵ -KO mice. In addition, we observed increased phosphorylation level of PKC δ/θ and dysregulation of insulin signaling in DGK ϵ -deficient WAT. On the other hand, no alteration in phosphorylation level of nPKCs and insulin signaling was found in liver and skeletal muscle. These data show that insulin sensitivity is decreased in a tissue-specific manner in DGK ϵ -KO mice under HFD-feeding conditions. DGK ϵ is shown to localize to endoplasmic reticulum, where TG is synthesized from DG. It may be hypothesized that accumulated DG activates PKC δ/θ , which leads to tissue-specific insulin resistance in WAT. Present study suggests that DGK ϵ plays a crucial role in glucose and lipid metabolism in WAT and its deletion increases susceptibility to HFD-induced obesity. Potential link between DGK ϵ and glucose and lipid metabolism will be fundamental in understanding the prevention and treatment of metabolic diseases.

P2400**Board Number: B1083****The effect of interleukin 1 β on VLDL secretion in steatotic hepatocytes during defatting.**A. Chen-Liaw¹, G. Yarmush², F. Berthiaume²;¹Biochemistry, Cell and Molecular Biology, Univ Scranton, Morris Plains, NJ, ²Biomedical Engineering, Rutgers University, New Brunswick, NJ

Steatotic livers complicate liver transplantation due to their increased susceptibility to primary non-function when exposed to ischemia reperfusion injuries which are inherent in all transplants. To combat this, one option is to perfuse, *ex vivo*, the steatotic livers with a defatting cocktail that reduces lipid droplets accumulated within hepatocytes. The current defatting cocktail functions by upregulating β -oxidation to break down free fatty acids. Additionally, experiments implementing the defatting cocktail have shown minimal levels of secretion of very low density lipoproteins (VLDL) from hepatocytes. Therefore, another synergistic approach involves the reduction of triglycerides (TG) via VLDL secretion from liver cells. This research project investigates the effects of interleukin 1 β (IL-1 β), a proinflammatory cytokine, on VLDL secretion in steatotic hepatocytes during steatosis-reduction treatments. To investigate the role of IL-1 β in VLDL secretion, we cultured a human hepatocyte cell line (HepG2/C3A) in media (Dulbecco's Modified Eagle Medium) supplemented with linoleic acid and oleic acid for two days, thus inducing steatosis. Cells were then switched to media supplemented with IL-1 β , the current defatting cocktail, or the combination of both. The defatting cocktail consisted of: amino acids, forskolin, visfatin, hypericin, scoparone, L-carnitine, GW7647, and GW501516. Intracellular TG content as well as that released in the medium were measured with Nile Red staining and with biochemical TG assays. Hepatocytes treated with IL-1 β alone yielded the smallest reduction in TG content. This may be attributed to increased VLDL secretion. TG reduction was greater in hepatocytes treated with the defatting cocktail and greatest in hepatocytes treated with the defatting cocktail + IL-1 β . These results suggest that although IL-1 β increases VLDL secretion, it may also inhibit β -oxidation pathways activated by the defatting cocktail. Future mechanistic studies will investigate the cellular pathways underlying the activity of IL-1 β . A more profound understanding of its mechanisms may pinpoint future molecular targets for new defatting agents that will increase the efficiency of *ex vivo* defatting therapies for steatotic donor livers.

P2401**Board Number: B1084****Decreased expression of SR proteins alters lipid droplet morphology and triglyceride levels in *Drosophila* larvae.**S. Ng¹, B. Kakrecha², A.A. Nagengast¹;¹Biochemistry, Widener University, Chester, PA, ²Biology, Widener University, Chester, PA

Obesity is a growing epidemic in the United States, and it is influenced by nutrition, lifestyle, and gene regulation. Obesity is characterized by an increased BMI as well as a high lipid profile. Triglycerides are stored in lipid droplets within fat cells. Previous research shows that lipid droplet morphology is altered in response to reduced expression of spliceosomal proteins and SR proteins. SR proteins are serine-arginine rich proteins that enhance splicing of weaker splice sites through exon definition and intron bridging, and they play a vital role in recruiting the spliceosome for alternative splicing. This project focuses on the relationship between lipid storage and the SR protein profile expression. Reduced expression of the SR protein 9G8 has been shown to decrease triglyceride storage in larvae of *Drosophila melanogaster*. Testing of additional SR proteins shows that decreased expression of RSF1, SF2, and hnRNP significantly altered triglyceride levels compared to controls. The SR protein profile is being characterized at different nutrient conditions using a Refeeding Assay and Western Blots with commercially available antibodies including phosphor-specific antibodies. This project will identify key SR proteins in triglyceride storage and potentially obesity, as well as characterize the change in SR protein expression and post-translational modifications as a result of changing nutrient conditions.

P2402**Board Number: B1085****Decreased expression of the SR protein 9G8 and sex determination genes alter lipid droplet morphology and triglyceride levels in *Drosophila* larvae.**J. Iman¹, G. Manilal¹, B. Kakrecha¹, A.A. Nagengast²;¹Biology, Widener University, Chester, PA, ²Biochemistry, Widener University, Chester, PA

The storage of lipids is a tightly conserved process that is important to the survival of all living organisms. The fat body of *Drosophila* responds to different nutrient conditions and controls overall energy metabolism by regulating long-term storage of triglycerides in structures called lipid droplets (LDs), thereby serving a function similar to liver and adipose tissue in mammals. Previous genome-wide RNAi screens in *Drosophila* cells identified mRNA splicing factors as playing a role in LD formation; their decreased expression results in the production of fewer LDs. Using RNAi knockdown experiments under GAL4-UAS control in the fat body of larvae, we have identified several splicing factors that control lipid storage *in vivo*. Interestingly, knockdown of the SR protein 9G8 in the larval fat body is male lethal, decreases triglyceride levels and alters lipid droplet morphology. However, knockdown of sex determination genes alone does not affect triglyceride levels although double mutants of 9G8 and the

sex determination genes had a more drastic LD and triglyceride level phenotype than 9G8 alone. We are currently in the process of identifying the metabolic targets of 9G8 activity that results in decreased triglyceride storage.

P2403

Board Number: B1086

Hypolipidemic effects of *Eryngium carlinae* in streptozotocin-induced diabetic rats.

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Patients with diabetes mellitus (DM) are at increased risk of suffering atherosclerotic cardiovascular diseases. Result of hyperglycemia and insulin deficiency has disturbances in lipid metabolism, as reflected high levels of triacylglycerides (TAG) and low-density lipoprotein-cholesterol (LDL-C) and low levels of high-density lipoprotein-cholesterol (HDL-C) in serum. Previously, we reported that the ethanol extract of *Eryngium carlinae* was able to normalize the levels of total cholesterol and TAG in diabetic rats. This study fractionates and analyses the extract composition with the purpose of identifying potential compounds responsible for hypolipidemic effect. Determinations were performed on blood lipid profile and analyzed the activity of HMG-CoA reductase and the LDL receptor levels (LDL-r), comparing our results with a hypolipidemic drug, atorvastatin. The results indicated that the extract of *E. carlinae* decreased triacylglycerides, total cholesterol, non-HDL cholesterol and increased HDL cholesterol levels in the serum to levels equal or even better than atorvastatin. The determinations of the activity of HMG-CoA reductase and LDL-r levels in diabetic rats were not modified by the extract consumption, indicating that the hypolipidemic effect observed was not through the inhibition of the cholesterol biosynthesis as is atorvastatin, but possibly acting at the intestinal absorption level. In this study we reported that one of the identified compounds in the extract is stigmasterol, and it decreases the absorption of dietary cholesterol by competing with it. However, the exact mechanism should be clarified in later works.